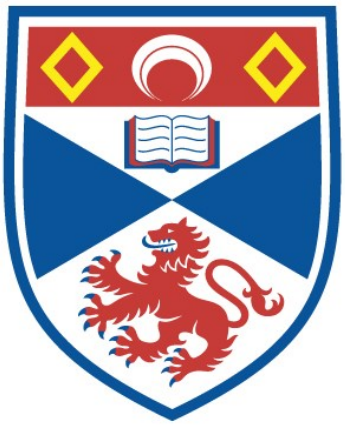
STUDIES ON BACTERIAL AMYLASES

William Macdonald Ledingham

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1968

STUDIES ON BACTERIAL

α-AMYLASES

by

William Macdonald Ledingham, B.Sc.

A thesis

submitted to the University of St.Andrews

in application for the Degree of Doctor

of Philosophy

Biochemistry Department,
The University,
St. Andrews.

May, 1968

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry of the United College of St. Salvator and St. Leonard, St. Andrews, under the direction of Dr. Stephen Bayne.

C E R T I F I C A T E

I hereby certify that William Ledingham has spent nine terms in research work under my direction, and that he has fulfilled the conditions of Ordinance No.16 (St. Andrews) and that he is qualified to submit this thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October 1959, and graduated with the degree of Bachelor of Science, Second Class Honours in Biochemistry in June 1963.

In October 1963, I was awarded an Agricultural Research Council Studentship to study Microbiology under the direction of Professor S. R. Elsden in the Department of Microbiology, University of Sheffield.

In October 1964, I was appointed Assistant Lecturer in Biochemistry in the University of St. Andrews and matriculated as a research student.

ACKNOWLEDGMENTS

Grateful thanks are extended to my supervisor Dr. Stephen Bayne for his interest and criticism throughout this work. I should also like to thank Dr. W. B. Hornby for his helpful advice and encouragement concerning the CM-cellulose-amylase experiments. Thanks are also due to Mrs. McAlister for typing and Mr. W.J. Blyth for photography.

C O N T E N T S

CONTENTS (Contd.)

CONTENTS (Contd.)

CONTENTS (Contd.)

CONTENTS (Contd.)

CONTENTS (Contd.)

INTRODUCTION

1.1 Amylase activity has been recognised for many centuries in Japanese fermentation processes and amylases were amongst the earliest enzymes to be studied from a biochemical point of view. Their importance today lies in their ability to convert starch and other amylaceous polymers into fermentable sugars, this conversion being the basis of the brewing and distilling industries.

Amylases, widely distributed in animals, plants and bacteria, fall into three broad classes. α-Amylases, (α 1,4-glucano 4-glucanhydrolase, EC 3.2.1.1) which are calcium metallo-proteins, catalyse a rather random hydrolysis of starch-type polymers causing a rapid fall in viscosity and iodine-staining power of the polymer. The intermediate products are oligosaccharides of varying sizes while the final products are maltose, D-glucose and maltotriose together with branched oligosaccharides containing the original α-1,6-inter-chain linkages.

β-Amylases are sulphydryl-containing proteins, found in some plants, which catalyse the stepwise hydrolysis of the penultimate glycosidic linkages of the outer chains with the production of maltose. This hydrolysis is interrupted by the presence of α-1,6-linkages leaving a large molecule termed a β-limit dextrin which is resistant to further β-amylase action.

Glucoamylases or γ-amylases are found in certain moulds and catalyse the stepwise liberation of D-glucose from amylaceous polymers.

This thesis is concerned only with the α-amylases and in particular those produced by strains of the spore-forming aerobic micro-organism Bacillus subtilis.

Peltier and Bechard (1945) isolated over 200 strains of an organism which they classified as a Bacillus subtilis which were capable of amylase production as shown by their ability to hydrolyse starch when grown on starch-agar plates.

There were wide variations in the nature of their amylolytic action. One isolate appeared to have greatly increased saccharifying activity as compared to its dextrinizing activity. Many isolates were found to be sensitive to a protein amylase inhibitor found in wheat. Strains from ropy bread were found to be insensitive to this inhibitor. Keen ~~et al~~ and Bechard. (1946) attempted a classification based on these criteria:

(i) B. subtilis saccharifying types - formed by strains isolated mainly from plant material.

(ii) B. subtilis non-saccharifying types - strains isolated from ropy bread. These amylases are similar in

their characteristics to commercial amylases.

(iii) Bacillus polymyxa type of amylase - B. polymyxa produces a type of amylase which shows characteristics of both α-amylase and β-amylase.

(iv) Bacillus macerans type of amylase - B. macerans produces an amylase which catalyses the conversion of starch to cyclic Shardinger dextrins (see French, 1957). These dextrins are non-fermentable but are slowly transformed into fermentable sugars by the macerans enzymes. It is now known that B. macerans produces a specific cyclodextrinase. [DePinto and Campbell (1968)].

1.2 Production of amylase by growing cultures of bacteria

The synthesis of α-amylase by growing cultures of B. subtilis has been studied by a number of workers and the basic conditions for production in high yields are known [Hagihara (1951); Fukomoto (1957)]. For amylase production in quantity, a carbohydrate source is required as well as a nitrogen source together with a basic mineral requirement. The carbohydrate may be any of a variety of sugars, some less effective than others, or one of the

amylaceous polymers, e.g. starch, amylose, amylopectin. Nitrogen may be supplied in the form of either an ammonium salt or a complex organic source.

Coleman and Elliot (1962), studying the formation of α-amylase in growing cells of B. subtilis showed that in the absence of any added nitrogen source growth was very slow, the logarithmic phase commencing only after some 60 hours of incubation. Accumulation of enzyme in the medium began at the beginning of the logarithmic growth phase and paralleled the growth rate from approximately the middle of this phase. Growth in a medium containing an exogenous supply of nitrogenous material (nutrient broth in this case) was naturally much faster. In this case the appearance of extracellular amylase lagged behind the cell growth rate and reached a maximum only after the termination of the logarithmic phase.

These findings of Coleman and Elliot (1962) are not at all in agreement with those of Nomura and Hosoda (1958) who claim that α-amylase is not synthesised at all by B. subtilis in the logarithmic phase of growth. The latter authors suggest that synthesis takes place during the stationary phase, after cell multiplication has

normally ceased, as a type of abnormal cellular biosynthesis.

Nomura and ~~his colleagues~~ Hosoda (1958) suggest that stationary phase synthesis of amylase and a phenomenon known as 'anaerobic lysis' are intimately connected in B. subtilis. They advance the idea that cells of B. subtilis producing amylase in the stationary phase are highly unstable and rapidly undergo lysis. Nomura (1958) states that anaerobic lysis takes place in an amylase-producing culture whenever anaerobic conditions supervene; these are promoted by inhibitors of cellular respiration such as cyanide or azide. Anaerobic lysis is an insignificant phenomenon when cells are grown on media which do not promote amylase formation. From the lysates, a lytic enzyme, whose site of action is the cell wall, has been isolated.

The action of this lytic enzyme is necessary for release of amylase molecules into the growth medium, depolymerisation of the cell wall occurring in stationary phase cells actively producing and ~~excreting~~ liberating amylase, ~~causes~~ causing lysis of the cells. In further support of this hypothesis Nomura, Maruo and Akabori (1956) have studied the production of amylase in media of very high osmotic

pressure. High concentrations of sucrose or polyethylene glycol almost completely inhibit lysis of B. subtilis amylase-producing cells. In such cells depolymerisation of the cell wall still occurs but the exposed protoplasts are protected from osmotic destruction by the high concentrations of sucrose and polyethylene glycol and permit a more prolonged period of amylase synthesis. Similarly the high amylase levels in bacterial cells grown in the presence of high concentrations of starch are claimed to be due to the osmotic protective effect of the starch, which allows the cells to continue producing amylase over a longer period.

The above hypothesis is not, however, confirmed by the work of Fukomoto 等. (1957), who, supported by Coleman and Elliot (1962) have shown that amylase is produced by B. subtilis during the logarithmic phase of growth approximately in parallel with cell mass.

Welker and Campbell (1963a,b,c,d) have made a fairly detailed study of the α-amylase of Bacillus stearothermophilus. This bacterium produces a heat-stable low molecular weight amylase. These workers have shown, in agreement with Coleman and Elliot (1962) and Fukomoto's (1957) work on B. subtilis, that the

enzyme is synthesised and released during the logarithmic growth phase. A study of the effect of the addition of various carbon sources to a basic nitrogen-containing inorganic salts medium on the levels of enzyme production was made. In the case of certain sugars, e.g. glucose and sucrose, there appeared to be an inverse correlation between the rate of growth on the carbon source and the level of enzyme production. This relationship did not hold for starch and maltose. The considerably greater levels of enzyme produced in the presence of the latter carbon sources may have been due to a stimulatory effect, possibly inductive, of trace impurities such as members of the malto-oligosaccharide series. Pure grades of maltose had reduced stimulatory effects compared with technical grades. Welker and Campbell showed that the addition of pure malto-oligosaccharides to the basal medium did in fact induce amylase formation above the basic level produced in a sucrose medium. Maltopentaose showed the maximum inductive effect in this respect with a gradual decrease of inductive effect down the series of oligosaccharides to maltose.

Studies with gratuitous inducers (inducers which are not metabolised) in B. stearothermophilus [Welker and Campbell (1963d)] showed that phenyl-, ethyl-, and methyl-

α-D-glucosides are good inducers of α-amylase. They found that at a concentration of 10^{-3}M the above compounds promoted up to a three-fold increase in the amount of amylase synthesised. From these studies it appears that while a basic level of amylase production exists on media supplemented with glucose, sucrose or glycerol, it is possible, in B. stearothermophilus, to induce the formation of greater amounts with gratuitous inducers such as the above-mentioned glucosides or non-gratuitous inducers such as members of the malto-oligosaccharide series. This amylase may then be regarded as a 'partly constitutive' enzyme. (Welker and Campbell, 1963b)

1.3 Production of amylase by washed cell cultures of bacteria

In view of the claims of Nomura and Hosoda *et al.* (1958) for bacterial amylase synthesis in stationary phase cells, several authors have studied the synthesis of the enzyme in washed cell preparations harvested in the late logarithmic phase of growth.

Welker and Campbell (1963d) established that B. stearothermophilus washed cell suspensions in aerated

phosphate buffer released small quantities of amylase into the medium. The addition of an inducer stimulated amylase formation and the cells were found to produce it in amounts comparable with growing cells in the presence of the same inducer. The rate of formation levelled off after about 60 minutes; cells examined at this time were found to have their intracellular amino acid pools depleted of certain amino acids. The addition of casein hydrolysate to the cells restored enzyme-forming ability for a further period. The prior incubation of B. stearothermophilus cells with fructose, which causes rapid depletion of intracellular amino acid levels, markedly reduces amylase formation in cells subsequently incubated with an inducer. Similar results were reported by Eisenstadt and Klein (1961) who studied the synthesis of amylase in washed cell preparations of Pseudomonas saccharophila. Prior starvation of the cells appeared to deplete aspartic acid and glutamic acid and enzyme synthesis ceased.

The work of Coleman and Elliot (1962) on B. subtilis reveals many similarities in the pattern of induced enzyme synthesis in that micro-organism and in B. stearothermophil While studies on B. stearothermophilus [Welker and Campbell (1963a)] were confined to maltose and a number of

gratuitous inducers, Coleman and Elliot (1962) studied the effect of a wider range of carbohydrate sources on washed cell enzyme synthesis. A number of sugars stimulate synthesis by factors of up to 4-[redacted], glucose seems to have variable effects depending on the concentration and seemed to stimulate enzyme synthesis most if added in small repeated doses.

The effect of added nitrogen sources in B. subtilis gives results which are more complex and difficult to interpret. Ammonium ions were found by Coleman and Elliot (1962) to stimulate enzyme synthesis whereas, contrary to the results with B. stearothermophilus, the addition of casein hydrolysates had inhibitory effects. The addition of individual amino acids caused less inhibition than the mixture of casein amino acids. Nomura (1958) also found an inhibitory effect of casein hydrolysates on enzyme production. Fukomoto (1957), however, reported a stimulatory effect but his experiments were carried out over a much longer period of time. This apparent diversity of results quite possibly stems from membrane transport differences arising from variations in experimental methods used in the preparation of washed cells. In mixtures of amino acids there may be

competition between individual amino acids for acceptor sites on the amino acid membrane transport systems. This could easily result in an intracellular spectrum of amino acid concentration quite different from that prevailing extracellularly.

Coleman and Elliot (1962) noted that incubation of washed cell cultures of B. subtilis in an atmosphere of nitrogen totally inhibited production of amylase, suggesting that a supply of respiratory energy was required for its formation. This they confirmed by showing that dinitrophenol, an uncoupler of respiratory chain phosphorylation, could produce a similar inhibition. Also, chloramphenicol, a specific inhibitor of certain stages of protein synthesis, inhibits amylase production in both growing cells and stationary washed cells [Coleman and Elliot (1962); Welker and Campbell (1963d)]. This, together with Coleman and Elliot's (1962) observations on inhibition by p-fluorophenylalanine (an analogue of phenylalanine incorporated into enzyme protein during biosynthesis) seems to indicate a de novo synthesis of amylase protein from small molecules rather than, as had been previously suggested for stationary phase formation, conversion of amylase precursor into the active enzyme protein. [Nomura, Maruo and Akabori (1956)].

It has been known for a number of years that a significant fraction of the protein of non-growing bacterial cells undergoes turnover. Rickenberg and Lester (1955) reported that a mutant of E. coli induced to form β-galactosidase under non-growing conditions, produced up to 5% as much enzyme as when induced under logarithmic growth conditions. This represented synthesis of the enzyme from components of other cellular proteins undergoing turnover as there was no net increase in protein. Eisenstadt and Klein (1961a) have compared the formation of amylase in cultures of Pseudomonas saccharophila growing in starch with washed cells of the same organism with starch added as inducer. By measuring the incorporation of labelled amino acids into amylase protein during growth on starch as carbon source, they found that amylase protein constituted some 0.08% of cellular protein in such cultures. A relatively much higher level of label incorporation into amylase protein was found in the washed suspensions induced with starch. Since the total amylase production was essentially equal in growing and non-growing cells, general cellular protein synthesis is reduced in non-growing cells while amylase synthesis continues at the same rate as in growing cells. Thus P. saccharophila selectively synthesises amylase protein under these conditions.

1.4 The purification of bacterial amylases

1.4.1 Crude culture filtrates

Early reported work on production of amylase in quantities suitable for purification involved incubation of cultures for many days, sometimes even weeks (Tilden and Hudson, 1942). Frequently amylase levels were reported to reach maxima in culture fluids only after 10-14 days. In retrospect, such lengthy incubations were probably the result of non-ideal growth media (e.g. oatmeal, maize meal suspensions and various other complex cereal mixtures) and a lack of appreciation of the physiological needs of the bacteria for rapid growth and consequently earlier enzyme production.

With the advent of commercially available bacteriological media and nutritionally-standardised components, media of defined chemical composition have been used and most recent work on amylase-producing bacteria is based on the use of starch in one form or another as the carbon and energy source and inducer of amylase synthesis. Maximal levels of enzyme were reported to be reached within 24 to 60 hrs. with such media (Campbell, 1955).

Most experimenters consider α-amylase produced by Bacillus species to be a predominately extracellular enzyme and consequently purification is from the supernatant fluid

after growth. Bacterial cells are removed either by centrifugation or by filtration using such filter aids as celite (diatomaceous earth). These processes will also remove any remaining insoluble amylaceous material derived from the medium itself.

1.4.2 Ammonium sulphate fractionation

In order to reduce amylase-containing culture filtrates to easily manageable volumes, precipitation of crude enzyme with ammonium sulphate has been widely used. Most workers have found that 50% saturation with ammonium sulphate is a sufficient concentration to precipitate most of the amylase protein from crude culture filtrates. Campbell (1955), after precipitating with ammonium sulphate at 100% saturation, extracted B. stearothermophilus amylase with 1% NaCl. This gave an amylase preparation with some 100-fold increase in specific activity over the crude filtrate. In later work, Manning and Campbell (1961) used 20% saturation with ammonium sulphate together with 20% saturation with sodium sulphate and achieved only a 28-fold increase in specific activity. Robyt and French (1964) found that 50% saturation with ammonium sulphate yielded a similar 28-fold increase in specific activity in their purification

of B. polymyxa amylase. Schwimmer and Balls (1949) achieved only a 15-fold increase with ammonium sulphate saturation to 43% in their purification of α-amylase from a commercial malt extract.

An alternative to ammonium sulphate precipitation preferred by some authors [Babbar, ~~et al.~~ Powar and Jagannathan (1962)], [Robyt and French (1964)], is to reduce the culture volume by as much as 50% by rotary evaporation at a temperature of approximately 30°C.

1.4.3 Organic solvent precipitation

As an alternative to ammonium sulphate precipitation amylase may be precipitated by a variety of organic solvents. Amylase loses little enzymic activity on precipitation from 50% ethanol provided the temperature is kept in the range 0-5°C. Campbell (1955) attempted to purify further his ammonium sulphate fraction of B. stearothermophilus amylase by two precipitations with cold acetone but achieved only a 2-fold increase in specific activity. Later experiments [Manning and Campbell (1961)] achieved a 6-fold increase with the same enzyme. Babbar et al.(1962), describing a rapid method for the crystallisation of B. subtilis amylase, evaporated the culture volume down to 50% of its original

volume and precipitated the protein with an unspecified quantity of ethanol. A later stage involved the precipitation of the enzyme in 50% ice-cold acetone. Although details are not given of the purification achieved by this particular stage, it could not have been greater than 2-fold. Robyt and French (1964) used tertiary butanol to obtain a 2-fold purification with an ammonium sulphate fraction of B. polymyxa amylase, while a subsequent fractionation in propanol achieved a further 6-fold purification.

Caldwell et al. (1952) describe a 7-stage purification of pig pancreatic amylase involving 3 ethanol-ether or ethanol-phosphate fractionations. No details are given of the purification factor but it is claimed that some 70-80% of the original activity was retained in the final purified enzyme powder.

Fractionation with organic solvents is clearly a less effective procedure than salt fractionation. As an alternative to the latter it is probably justifiable but, used after salt fractionation, it appears to achieve little additional purification and could be omitted in favour of earlier crystallisation attempts or starch-glycogen absorption purification.

1.4.4 Crystallisation of amylase

As well as being amongst the first enzymes to be studied from a biochemical point of view, amylase was one of the earlier enzymes to be crystallised (Meyer, 1947). Meyer, starting with a commercial preparation of B. subtilis amylase, purified the enzyme by ammonium sulphate fractionation and solvent precipitation and crystallised the enzyme from distilled water at 4°C overnight. Further re-crystallisations carried out from sodium acetate solutions at pH 5.6 resulted in a final crystalline material with a specific activity some 60-fold greater than the starting material. A method of Stein and Fischer (1961) for B. subtilis amylase purification involves crystallisation in the cold from calcium acetate solution, pH 6.8, with the addition of a trace of zinc ions (B. subtilis amylase molecules form dimers with the involvement of zinc ions). Babbar et al. (1963) report a rapid method of B. subtilis amylase purification involving crystallisation from 0.1M calcium acetate solution, pH 6.8.

1.4.5 Purification by specific enzyme-substrate complex formation

Specific enzyme-substrate complex formation, if such complexes can readily be removed from solution, has

become a powerful tool in enzyme purification procedures. Erlanger (1958) suggests that if a molecule has the structural requirements to be either a substrate or competitive inhibitor for a specific enzyme and can be chemically modified to introduce hydrophobic groups to render it water-insoluble, then it has potential as a means of purifying the enzyme. He demonstrated this in the case of chymotrypsin by studying the interaction of the enzyme with the insoluble competitive inhibitor N-carboxybenzyloxy-L-leucyl-D-phenylalaninebenzyl ester. Other proteolytic enzymes were found to be only minimally absorbed to this substance.

Amylases and related enzymes are especially amenable to this type of purification while modification of the substrate is unnecessary as the amylase-polymer complex can be readily precipitated by 40% aqueous ethanol, at which concentration the enzyme is relatively stable. Some 60 years ago Starkenstein (1910) showed that 'liver amylase' was absorbed by a suspension of insoluble starch from aqueous solution. Later, various authors showed the same effects with amylases from pancreas, saliva and malt. Holmberg (1933) showed that a separation of β-amylase and α-amylase was possible using

starch as adsorbent in the presence of maltose. Maltose, the major end-product of β-amylase action, inhibited β-amylase binding to the starch. Starches from many sources have been tested for their efficiency as adsorbents [Schwimmer and Balls, (1949b)]. Results indicate that variations in adsorption efficiency may depend on a number of factors. With starch granules the surface area of the granule parallels the adsorptive power. Certain treatments of starches increase adsorptive power e.g. mild acid digestion as is used in the preparation of some 'soluble starches'. Starch fractions (amylose and amylopectin) have greater adsorptive power than do the granules and this is dependent on the structure of the fraction (and hence the method of isolation) as well as the source. One thing that adsorption is not affected by is the degree of purity of the amylase preparation, from which adsorption is taking place. Adsorption was also found to be a function of time and adsorption efficiency was measured on the basis of the amounts of enzyme adsorbed during a period of 2 minutes. Adsorption was found in some cases to continue for as long as half an hour.

Starches suffer from the disadvantage that they are ill-defined complex aggregates which are neither pure

nor homogeneous. This presents difficulties in elucidating the nature of the complex formed and the kinetics of its formation. For this reason later work on complex formation has been directed to glycogen-amylase complexes. Binding to glycogen has been observed for several different enzymes. Leloir and Goldenberg (1962) found that, during the purification of glycogen synthetase from fractions of rat liver homogenate, the enzyme was bound to 'particulate' glycogen. Similarly bound phosphorylase could be removed by washing in saline buffers containing soluble starch. Sutherland and Wosilait (1960), studying the purification of dog liver phosphorylase, also found that phosphorylase tended to accompany glycogen during purification. Ratios of twenty parts of carbohydrate to one part of protein were still found after several ammonium sulphate fractionations and ethanolic precipitations. These authors separated the two components by adsorption on calcium phosphate gels whence the protein with only minimal traces of carbohydrate could be eluted by citrate buffer.

Loyter and Schramm (1962) have investigated the formation of complexes between amylase from pancreatic

and salivary glands and glycogen. Such complex formation requires less polymer than starch-enzyme formation and reaches equilibrium much more rapidly. The complexes are insoluble in 40% ethanol. They found that a definite ratio of enzyme to glycogen is optimal for enzyme precipitation. Complexes precipitated in 40% ethanol can be removed by centrifugation at low temperatures and then suspended at 30°C in aqueous buffers to allow removal of the glycogen by enzymic digestion to small oligosaccharides. Such a purification step should theoretically yield pure amylase as only amylase molecules should bind to the substrate (unless glycogen synthetase and phosphorylase are also present). In practice, amylase so purified has a specific activity equal to that of the best crystalline preparations. Analysis of the complex reveals it to be 0.5 mg. enzyme/ mg. glycogen. As the molecular weightSof these amylases are around 45,000 this means that one molecule of enzyme is bound by some 550 glucosyl residues. Loyter and Schramm (1962) consider that if an amylase contains more than one binding site then a lattice type structure could result from intermolecular linkages between glycogen molecules. This arrangement would give rise to a

macro-complex consisting of many molecules of both glycogen and enzyme. Levitski, Heller and Schramm (1964) have more recently shown that ethanol is not necessary to precipitate amylase-glycogen complexes. Spontaneous precipitation will occur in the cold in aqueous solutions. The complex has been shown to consist of amylase molecules bound to large molecular weight dextrins formed from slight enzymatic hydrolysis of the glycogen. Both excess enzyme and excess dextrins were shown to have an inhibitory effect on the formation of an insoluble complex. Again a lattice type of structure is proposed for the complex, and smaller dextrins, incapable of multivalent binding, would be expected to inhibit macro-complex formation. The above authors have shown that purified dextrins of DP (degree of polymerisation) less than 22 do not themselves form an insoluble complex with amylase and inhibit the formation of such complexes with higher dextrins.

A point that is of possible significance is that the removal of calcium from amylase prevents complex formation. Since calcium is also necessary for enzymatic activity it appears likely that binding in complex formation and enzymatic activity take place at

the same sites.

1.5 Specificity and action patterns of α-amylases

α-Amylases are generally regarded as endoenzymes hydrolysing in a somewhat random manner the α 1,4-links in glucans of the starch or glycogen type or compounds derived from these polymers. The final products are a mixture of low molecular weight oligosaccharides, D-glucose and occasionally a high molecular weight resistant α-limit dextrin. Within this rather broad framework there are considerable variations as to fine specificity and action pattern between amylases from different sources. Prior to the last decade amylase digestion studies were conducted using the polymer substrates, starch and glycogen, the fine structure of which were then unknown. The development of paper chromatographic and other methods for the separation of carbohydrate polymers and their degradation products led to the preparation of pure compounds of the malto-oligosaccharide series of known structure, compounds previously referred to collectively as 'dextrins'. Such defined substrates of amylases were indispensable tools in the elucidation of the specificity of the enzymes. Such data in turn

led to the use of amylases as tools for the investigation, with much success, of the fine structure of starches and glycogens.

1.5.1 Action patterns of amylases with respect to macromolecular substrates

Macromolecular substrates comprise the starches and the glycogens. α-Amylases characteristically catalyse rapid hydrolysis of the molecule to oligosaccharides with rapid diminution of viscosity and iodine-staining power. The outer chains are particularly susceptible to enzyme action and rapidly give oligosaccharides reflecting both the outer chain length of the polymer and the fine specificity of the particular amylase. Unlike β-amylase the action of which is restricted to the outer chains of the polysaccharide, α-amylase also attacks the interior linkages of the molecule. α 1,6-links are, however, totally resistant to amylase action and constitute an impediment to the action of the α-amylase. Such links persist as structural features of small branched oligosaccharides, the structure of which again depends on the specificity of the individual amylase. It has been found that in certain shell-fish glycogens [Heller and Schramm (1964)]

there exists regions of the molecule which are densely branched as to prevent totally α-amylase action. Such structures give rise to high molecular weight limit dextrins (α-macrodextrins) similar to those occurring after β-amylolysis of glycogen or amylopectin. α-Macrodextrins are probably derived from regions of the polymer molecule where the branch points are separated by less than 3 α 1,4-linked glucosyl residues. Heller and Schramm (1964) have reported that some 11% of a shellfish glycogen remains as an α-macrodextrin after α-amylolysis compared with very much smaller amounts, 2-3% in the case of rabbit liver and muscle glycogens, and about 1% in the case of phytoglycogen.

The presence of such densely-branched regions of the molecule is not necessarily apparent from determinations of degree of branching in the intact molecule as the methods which have been employed (non-reducing and group analysis) give <u>average</u> values for the whole molecule. Similarly, methods for analysis of the dextrins produced by amylase digestion frequently involved their elution from charcoal under conditions in which high molecular weight dextrins would not be eluted. Heller and Schramm (1964) fractionated dextrins produced by α-amylolysis by

Sephadex gel filtration and were able to detect macrodextrins which had D.P's of between 30 and 330. These macrodextrins were classified as α-amylase limit dextrins by Heller and Schramm because of their extremely slow rate of further hydrolysis by α-amylase. They estimate this as some 5000 times slower than the initial rate of hydrolysis of glycogen. This is probably because the few remaining linkages that are susceptible to α-amylase action are highly inaccessible to the enzyme. Heller and Schramm's studies have shown that branching in glycogens is not evenly distributed throughout the molecule and that densely-branched regions are more frequent in shell-fish glycogens than mammalian liver glycogens.

For α-amylase action on polymeric substrates three distinct action patterns are possible - single chain, multiple attack and multichain attack. In single chain action the enzyme forms a complex with a particular chain and reaction is catalysed along that chain in one direction or the other, forming the oligosaccharides for which the enzyme is product specific until the end of the chain is reached. The enzyme then forms a second complex with another portion of chain and the process is repeated. (See Figure 1.5.1)

ACTION PATTERNS OF α-AMYLASE

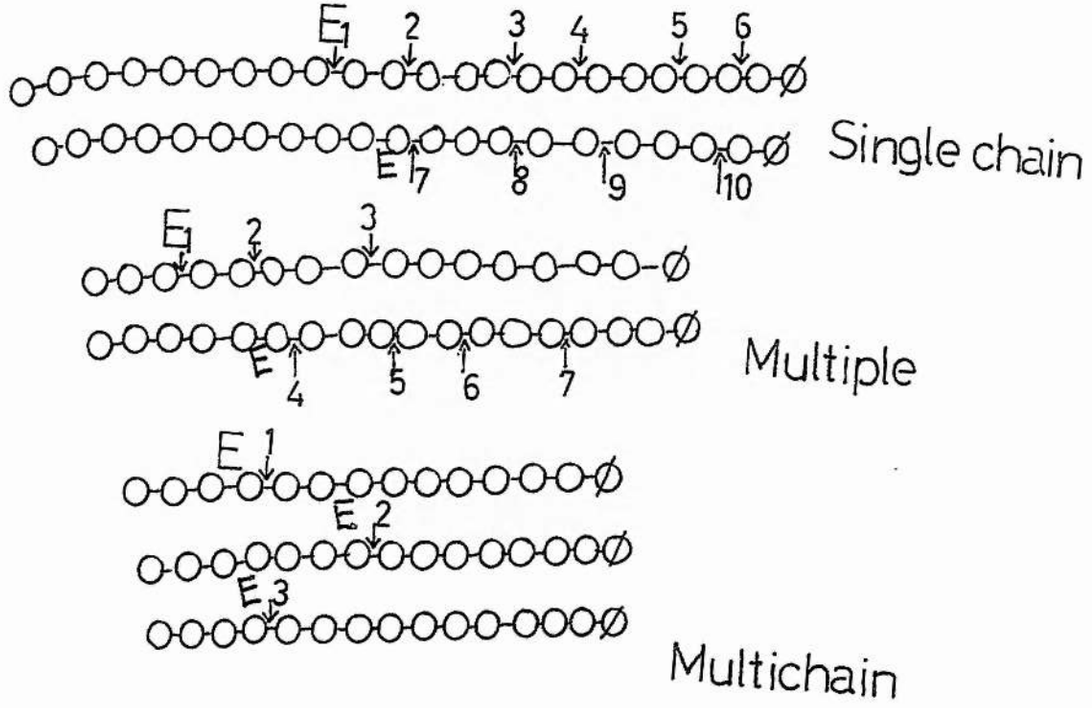


Fig 1.5.1.

At the other extreme is multichain catalysis where each effective enzyme substrate complex formation results in the hydrolysis of only one glucosidic bond. Between these two extremes lies the multiple attack mechanism of hydrolysis where each incident of enzyme-complex formation may result in the hydrolysis of several glucosidic bonds before the complex dissociates and a new complex is formed. The degree of multiple attack has been defined [Robyt and French, (1967)] as the average number of catalytic events, after the first, during the lifetime of the individual complex. This has been studied qualitatively by Kung 龔 鳳. (1963), and also by Robyt and French (1967), by plotting the fall in blue value (iodine-staining power) against the increase in reducing power as the digestion of amylose by amylases proceeds. Robyt and French (1967) investigated amylases from pig pancreas, human saliva and Aspergillus oryzae and found that the multiplicity of action decreased in that order at the optimum pH for each individual enzyme. Also of interest was their finding that the degree of multiplicity was dependent on the pH at which the reaction was carried out. Pig pancreatic amylase acting at pH 10.5 was found to act essentially

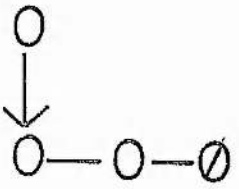
in a multichain manner. The fact that this enzyme, acting at pH 10.5, showed a lower reducing power for a given fall in blue value than acid-catalysed hydrolysis was taken by these authors to mean that acid hydrolysis involves a preferential attack on terminal glycosidic linkages producing somewhat more glucose than a purely random process would produce and also that the pancreatic enzyme under these conditions avoids such terminal linkages. Robyt and French (1967) have developed a quantitative method for the determination of the degree of multiplicity of action of amylases. Essentially this is based on the ratio of total reducing groups appearing during hydrolysis to reducing groups appearing in the polysaccharide fraction. Total reducing groups are the sum of those released by (a) primary attack on a polysaccharide molecule to give two other polysaccharide fragments and (b) secondary attack on the newly formed end of one of the fragments to give low molecular weight fragments. The polysaccharide fraction is easily separated by ethanol precipitation. The ratio of total reducing power to ethanol-precipitable reducing power gives a measure of the number of bonds broken per effective enzyme-substrate complex formed. The degree

of multiplicity is numerically equal to this number minus one (as the first bond broken gives a polymer fragment). Robyt and French (1967) report the respective multiplicities of action of pig pancreatic, human salivary and Aspergillus oryzae amylases as 6, 2, 1.7.

1.5.2 Action patterns of α-amylases with respect to oligosaccharides

The introduction of such techniques as multiple ascent paper chromatography and gel filtration separation of uncharged molecules have opened the way to studies of the interaction of α-amylase and the smaller oligosaccharides which are also substrates. Utilising these techniques pure maltosaccharides of the series from maltose to maltoheptaose can easily be prepared from partial acid-hydrolysates of starches or starch fractions. These small oligosaccharides, produced during the initial stages of amylase hydrolysis of macromolecules may be further hydrolysed to smaller fragments. The spectrum of oligosaccharides persisting at the end of this secondary and, in many cases, very much slower phase of α-amylase attack is usually characteristic of the particula

amylase and its product specificity. Walker and Whelan (1960) have studied oligosaccharide digestion by salivary α-amylase. This enzyme converts amylose rapidly into maltose and maltotriose and other oligosaccharides do not accumulate to any extent. Further secondary phase hydrolysis of maltotriose takes place but only very slowly at a rate some thousands of times slower than the initial rate on amylose. Walker and Whelan (1960) were able to establish that maltotriase activity was in fact a function of the amylase itself and not of a separate enzyme. These authors also described the smallest branched oligosaccharide (α-limit dextrin) which is formed from glycogen or amylopectin. This has the following structure:-



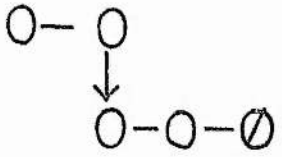
6^2-O, α-D glucopyranosylmaltotriose

The other species of α-limit dextrins remaining will depend on the fine structure of the polymer substrate

and may include α-macrodextrins of quite high molecular weight as previously described. These species of α-limit dextrins may not represent the completion of α-amylolysis as very high concentrations of enzyme may cause further hydrolysis. Studies with B. subtilis amylase [Robyt and French (1963)] have shown this α-amylase to be quite different from salivary amylase in its action on oligosaccharides. There seems to be a dual product specificity favouring the formation of maltotriose (G3) and maltohexaose (G6). Smaller amounts of maltotetraose (G4), maltopentaose (G5) and maltoheptaose (G7) also occur. Robyt and French (1963) studied the rate of further hydrolysis of these products with 10-fold increases in enzyme concentrations. Maltose (G2), G3 and G5 were not detectably hydrolysed at all at this enzyme concentration. G6 was converted slowly into G5 + glucose (G1) while G7 was more rapidly converted into G6 + G1 and G5 + G2. G6 and G7 appear to come from the exterior chains of the polymer as they are not produced at all when an amylopectin β-limit dextrin is used as substrate.

Using glycogen β-amylase limit dextrin as substrate no detectable oligosaccharides were produced

with *B. subtilis* amylase. This obviously reflects the short interior chain length in the glycogen molecule with insufficient linkages accessible to amylase action to permit fragmentation to small oligosaccharides. This observation is also in support of Heller and Schramm's (1964) reports of α-macrodextrin formation by pancreatic amylase digestion of various glycogens as mentioned above. The smallest branched oligosaccharide reported for *B. subtilis* amylase digestion of amylopectin or glycogen is a pentasaccharide of the following structure:



6^2-α D-maltosylmaltotriose

In contrast to salivary α-amylase, *B. subtilis* amylase produced from starch or glycogen considerable quantities of glucose, indicating its ability to hydrolyse terminal linkages. These differences in product specificity which result in oligosaccharide spectrum differences can therefore be used as a means of classifying amylases. Obviously the period of hydrolysis allowed is of importance in such investigations as very lengthy periods of digestion lead to the formation

of much the same products for all amylases (glucose, maltose and small branched oligosaccharides).

B. polymyxa produces an interesting type of amylase which has characteristics of both α- and β-amylases. B. polymyxa amylase produces β-maltose as the major end-product of polymer digestion [Robyt and French (1964)] cf. (α-maltose is the normal product of α-amylase action) and also catalyses the degradation of cyclic dextrins, an activity not associated with normal α or β-amylase action. Its action resembles α-amylase in its ability to hydrolyse internal chains. The most surprising feature of this enzyme is the high rate of attack on G6, which is degraded to predominantly G2. The rate of attack on G6 approaches that of attack on amylose by this enzyme and is greater than that on glycogen or amylopectin. Rapid G6 hydrolysis by this enzyme is in marked contrast to the slow hydrolysis of G6 by B. subtilis amylase. Cyclic G8 is hydrolysed to G2 without the appearance of any intermediate straight chain oligosaccharide; this suggests a multiple attack mechanism for hydrolysis. De Pinto and Campbell (1968b) have recently investigated the 'amylase' of B. macerans which is the other member of the Bacillus genus producing enzymes which degrade

cyclic dextrins. They have been able to separate the enzymic activity into an amylase which degrades starch into these cyclic dextrins but is unable to hydrolyse them and a cyclodextrinase which has no activity on starch but degrades cyclic G6, G7 and G8 with equal ease.

In view of De Pinto and Campbell's work (1968a,b), it may well be that greater purification of the B. polymyxa enzyme system would reveal two distinct activities, that of a β-amylase type resembling sweet potato β-amylase and a cyclodextrinase. However, Robyt and French (1964) report differential activity of their preparation of B. polymyxa amylase between cyclic G8 and cyclic G6, the latter being extremely slow, whereas B. macerans cyclodextrinase is equally active on both dextrins.

1.5.3 Diverse actions of bacterial amylases on mucopolysaccharides and related molecules

Over the past five years there have appeared a number of reports of amylase preparations hydrolysing certain (unspecified) linkages in mucopolysaccharide type molecules liberating mucopeptides, neuraminic acid-containing oligosaccharides and other hexose molecules

or, if not actually liberating any small molecules, then altering the structure of the macromolecule in some demonstrable way. Maley, ~~et al~~ McGavrachan and Delljiacco. (1966) have described the isolation of a glycosidically-linked glucosamine containing disaccharide following α-amylase hydrolysis of glycogen isolated from a galactosamine perfused liver.

A recent Japanese patent [Nishihara (1963)] describes the isolation of soluble collagen from ox-hide after pretreatment of the minced tissue with a bacterial α-amylase preparation followed by extraction of the protein with dilute acid. Greatly increased yields were obtained using this technique. Steven (1964) adapted this technique for the preparation of collagens from aged and rheumatoid tissues where normally only very small amounts of tropocollagen can be extracted with dilute acid and procedures such as thermal denaturation to the derived soluble gelatin or mechanical removal of the non-collagenous material must be resorted to. The reason for increasing difficulty of extraction as the tissue ages is thought to be an increase in the number of covalent linkages stabilising the collagen molecule. As a small amount of carbohydrate (0.5%)

is always associated with collagen [Hörmann (1960)], and this amount is found to be less in collagen solubilised with the aid of α-amylase treatment, it was naturally considered possible that α-amylase might be hydrolysing glycosidic bonds which were in some way involved in insolubilising the collagen molecule.

Glaeser and Mel (1966) have recently reported that the treatment of rat erythrocytes with bacterial α-amylase results in reduction of their anodic electrophoretic mobility with a concomitant release of a neuramino-oligosaccharide. On a re-investigation of this effect, Seaman, Jackson and Uhlenbruck (1967) confirmed the mobility change in the erythrocytes but were able to show that the sialic acid was released as part of a mucopeptide and not an oligosaccharide. This latter work highlights a real problem in assessing the effects of bacterial amylase preparations on complex structural systems, B. subtilis is known to produce a variety of proteolytic enzymes, the best characterised being subtilisin, as well as other carbohydrases. Complete purification of amylases is necessary if meaningful results are to be obtained.

While there has never been the slightest evidence or suggestion that an α 1,4-glucan chain (the normal amylase substrate) is involved in the structure of covalent crosslinks in the collagen molecule, the very slow but nevertheless finite rate of hydrolysis of maltotriose to glucose and maltose [Walker and Whelan (1960)] and the recent report of Yoshida (1967) of the hydrolysis of phenyl α-maltoside by amylase may be of significance when one interprets the effects of the amylase treatment on connective tissue. Steven (1964) used a 0.3% w/w solution of bacterial amylase for a period of 90 hours at room temperature. The amylolytic activity of such a preparation would be of the order of hundreds of thousands of units, quite sufficient to hydrolyse many linkages with an order of substrate affinity for the amylase akin to maltotriose. On the other hand the same considerations of time and enzyme concentration apply to the possible action of trace amounts of other enzymes, both proteolytic and carbohydrase in nature, which may be present in the preparation.

1.6 Aims of the present research

1) To investigate the biosynthesis of amylase by Bacillus subtilis on different media, under the influence of possible inducers, and under different growth conditions (batch and continuous culture), and its release as an extracellular enzyme. Also to investigate the effect of inducers on extracellular amylase release by washed cell suspensions.
2) To investigate purification techniques for the enzyme, especially glycogen-amylase complex formation.
3) To investigate some properties and action patterns of the enzyme and in this connection, the preparation and properties of CM-cellulose-α-amylase, an insoluble enzyme derivative.
4) To compare these properties with those of commercially available bacterial α-amylases claimed to be preparations from B. subtilis.

METHODS

METHODS

2.1 Microbiological Methods:

2.1.1. Maintenance of stock cultures

Cultures of *Bacillus subtilis* 3610 were obtained from the National Collection of Industrial Bacteria and were maintained on a medium 1% of starch and 0.4% Difco Yeast Extract, in 8 oz. screw-capped bottles at room temperature after overnight growth at 35°C on a metabolic shaker with vigorous shaking. The bacterium was subcultured at intervals of 2 months.

2.1.2. Experimental cultures

Experimental cultures were carried out in 500 ml. and 100 ml. Erlenmeyer flasks with working volumes of 200 ml. and 40 ml. respectively. The medium used was of the following basic composition:-

0.4% Difco Yeast Extract

the following salts each at a final concentration of 5 mg./litre: $FeCl_3$, $CaCl_2$, KCl, $MgCl_2$, $MnCl_2$, $Zn(CH_3COO)_2$, $Co(NO_3)_2$ [This is a simplified salts supplement based on that used for *Bacillus stearothermophilus* (Welker and Campbell, 1963a)].

This basic medium was supplemented with a carbon source depending on the experiment in question. Media

were sterilised at 15 psi for 20 minutes, except supplements of sugars which were sterilised by tyndallisation and added aseptically. Growth was at 35°C on a Gallenkamp metabolic shaker at 150 oscillations per minute. Inocula were 5-10% of the culture volume from a fresh culture in the same medium except where otherwise stated.

2.1.3. Large scale culture

Large scale culture, for enzyme production, was carried out in 10-litre and 20-litre Quickfit fermentation vessels with flanged lids fitted with silicone rubber tubes to permit aeration during growth. The vessels were sterilised by autoclaving at 20 psi for 2-3 hours. Small quantities of Silicone Antifoam A (I.C.I. Ltd.) were added to suppress frothing and the vessels were maintained at 37°C in water-baths. Inocula were carefully prepared for such cultures to permit a rapid 'take-off' of growth and consisted of 1-litre cultures of cells in the logarithmic phase of growth in the same medium. Under such conditions growth was maximal after 18-30 hours.

2.1.4. Harvesting of cells and preparation of cell-free supernatants

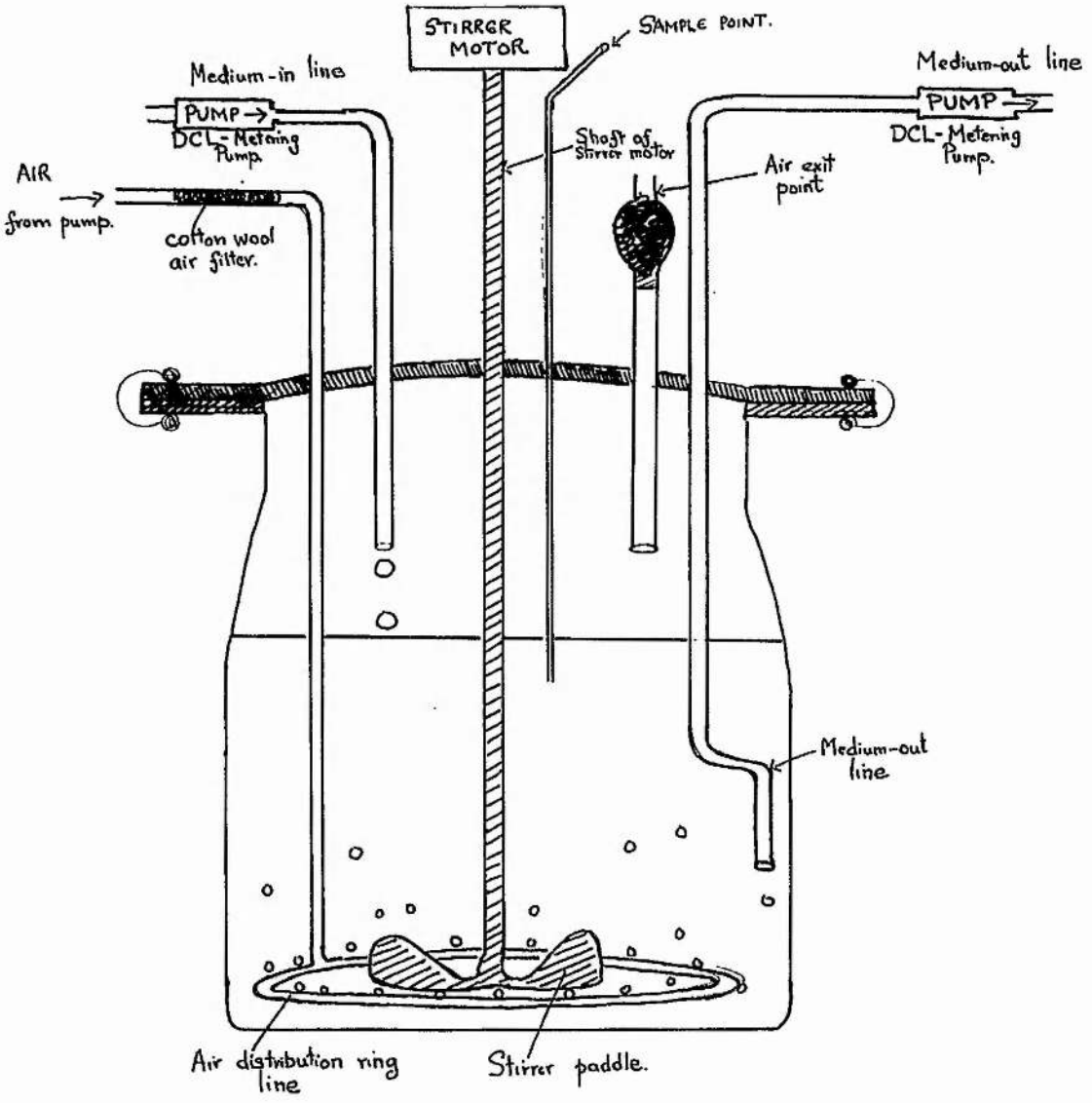
Cells and culture supernatants were separated by centrifugation at 5°C and 2000 g. When only the supernatant was required, filter aids such as celite (Hyflo) were used in conjunction with Buchner vacuum filtration. Celite was found relatively unsatisfactory, however, on account of its non-specific absorption of proteins (including amylase) and consequent reduction in yields of supernatant enzyme.

2.1.5. Continuous culture methods

A simplified continuous culture apparatus was constructed based mainly on the range of fermentation glassware available from Quickfit & Quartz Ltd. The reactor was a 2-litre capacity flat-bottomed fermentation jar (working volume 1.5 litres).

Through a multiple-necked flanged glass lid the reactor was equipped with the following:

1) A glass paddle stirrer with a flexible rubber coupling to a variable speed motor.
2) A multiperforated glass tube aerator arrangement connected to a Charles Austin Electromagnetic Air Pump providing oil-free air and equipped with a cotton-



REACTOR VESSEL Fig 2.1.5
FOR CONTINUOUS CULTURE

wool bacteriological filter in the air line.

3) A medium entry line permitting, dropwise, addition of medium to the culture.

4) A medium exit line permitting removal of media by pumping. The media lines were coupled to the two pumping heads of a DCL Micro Pump. The rates of pumping could be independently varied, hence enabling the working volume in the reactor to be kept constant.

5) A sterile sampling point through which small samples could be withdrawn by syringe from the reactor.

The sterile reservoir for medium and the collection vessel were 20-litre fermentation jars similarly equipped with flanged lids with provision for media lines and filtered-air entry or exit.

All pipeline was of either soft soda-glass or silicone-rubber tubing (blood transfusion grade) which stands repeated sterilisation. The reactor vessel was immersed during use in a large water-bath at 37°C. This maintained the reactor contents at a temperature of 35°C $\pm$ 1°C under conditions of constant aeration.

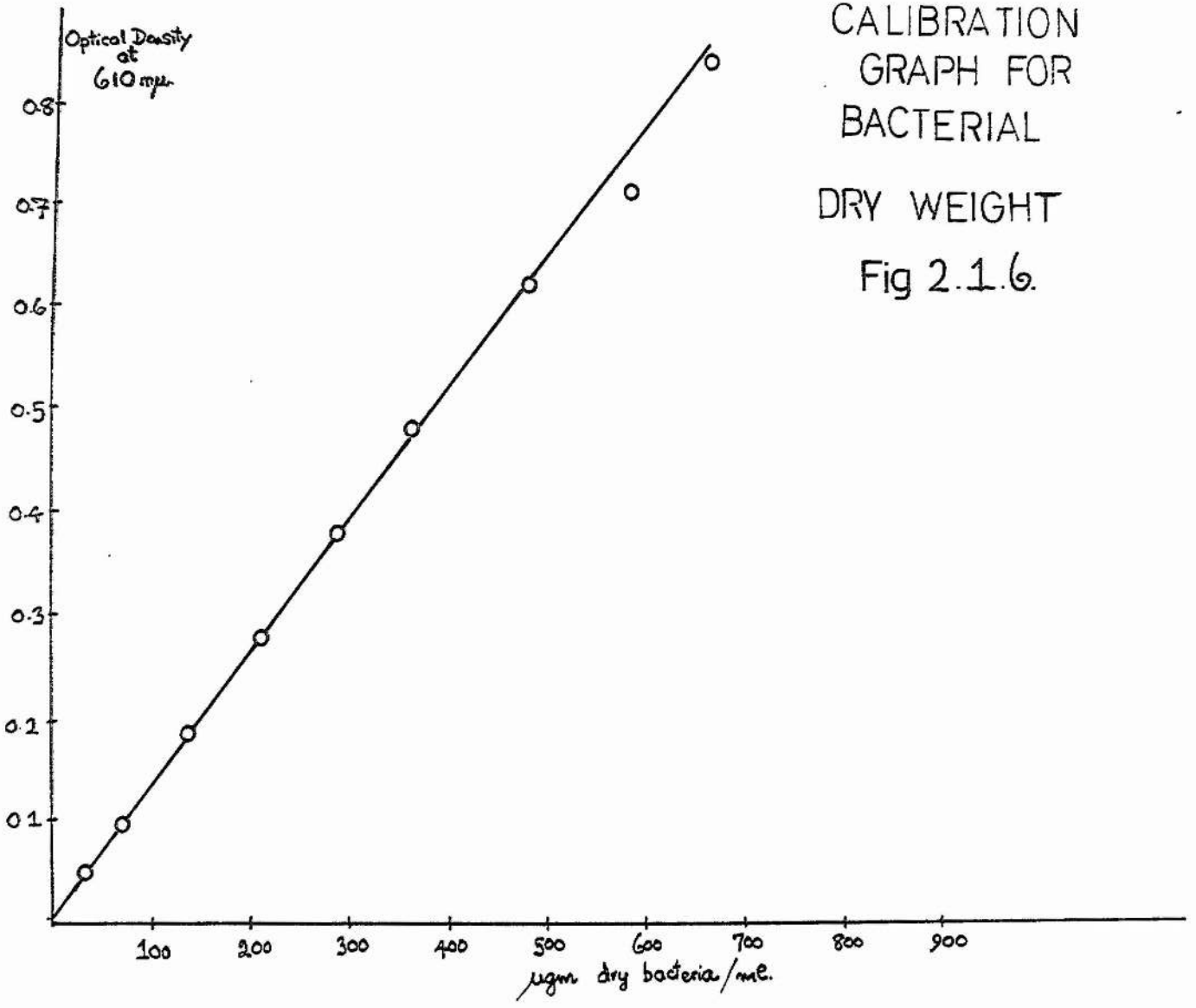
For sterilisation purposes the apparatus was autoclaved in sections. The complete reactor assembly

was autoclaved, media and air lines having been disconnected and sealed off and the stirrer shaft disconnected. The media supply and collection vessels were separately sterilised by autoclaving together with the pumping heads of the DCL Micro Pump at the beginning of each run. Further media supply vessels were aseptically connected as necessary.

This apparatus was unconventional in design in that culture fluid was pumped out of the reactor vessel and not removed via a constant level overflow device. This permitted the use of a water-bath for temperature control but necessitated a slight differential in pumping rates to correct for air bubbles in the liquid leaving the reactor. After initial technical troubles were overcome the apparatus was capable of functioning reliably over periods of a week or more. Sterile silicone antifoam was added from a syringe at intermittent intervals. As this compound was found to remain on the surface of the liquid and was hence not readily removed from the reactor vessel, frequent addition was not necessary.

3.1.6. Growth rate measurements

Bacterial growth was followed by optical density measurements at 610 mμ on a Unicam SP600 spectrophotometer.



CALIBRATION GRAPH FOR BACTERIAL DRY WEIGHT

Fig 2.1.6.

A calibration curve of optical density against dry weight of bacteria per ml. was prepared as follows:-

Bacteria were harvested from a 1-litre culture by centrifugation and washed with phosphate buffer, distilled water and finally resuspended in distilled water to form a thick suspension. Three 1 ml. samples from this suspension were dried to constant weight at 150°C. An average weight of dry bacteria per ml. was thus calculated. Optical density measurements were carried out on a series of dilutions of the thick suspension and a graph drawn of these optical densities against the calculated dry weight of the dilutions from the average value.

(See Fig. 2.1.6)

2.2 Biochemical and chemical methods

2.2.1. Assay of amylase

α-Amylase activity has been determined by numerous techniques over the present century, all being variations based on the following general methods:

1) Decrease in viscosity of a starch solution
2) Decrease in turbidity of a starch suspension
3) Decrease in 'blue value' of a starch-iodine complex
4) Increase in reducing power of a starch solution.

Viscometric and turbidometric methods which are simple assays not requiring much apparatus are largely now of historical interest only. Such is the variation in the physical properties of starch molecules that the reproducibility of these methods was poor.

The loss of 'blue value' of a starch-iodine complex is still a widely used method of amylase assay. According to Schoch (1961) the blue colour of the complex can be attributed to the lining up of iodine atoms inside the helical configuration which the polymer chains assume. The actual shade of colour depends on the number of turns of the helix, this depending on the chain length. The following table relates the colour of the complex to number of helical turns.

Number of helix turns	Colour of complex
less than 2	None
2	Brown
3-5	Red
6-8	Purple-red
9 or more	Blue

Activity can be related either to the time required to convert a given quantity of starch into a dextrin mixture giving no colour with iodine (the achromic point) or to the decrease in blue colour over a given time period. The former method is less accurate and no longer used. The latter method has been used throughout this work for the following reasons:

i) It is rapid and reproducible provided the enzyme concentration is within a range such that during the period of assay the fall in substrate concentration is not greater than 30%.

ii) Assay is based on the most rapid phase of α-amylase activity - the cleavage of the polymer into oligosaccharide chains.

The increase in reducing power of a starch solution is intrinsically the most accurate method as each cleavage of a glucosidic bond by the enzyme liberates

one free reducing group. It is, however, a more time-consuming assay than the iodine complex assay and was not used in this work.

Procedure for amylase assay

Reagents

(i) Buffer solution: 0.1M phosphate buffer, pH 5.7

(ii) Substrate solution: Amylose (BDH, prepared from potato starch) was used in preference to starch as it is more homogeneous. A 1% stock solution was prepared as follows:

80 ml. of 0.1M NaOH was heated to 90°C, removed from the heat and into it was poured a suspension of 1 g. of amylose in 5 ml. of ethanol, further traces of amylose being washed in with smaller amounts of ethanol. The ethanol boils off immediately leaving the amylose in solution. The solution was cooled and made up to 100 ml. From this stock solution a 0.1% substrate solution was made up with distilled water and stored at room temperature.

Solutions of amylose which showed a precipitate due to retrogradation were discarded.

(iii) Iodine-potassium iodide solution: A 1% stock solution was prepared by dissolving 30 g. potassium iodide in 250 ml. water, adding 12.7 g. of iodine and when the

iodine had dissolved, diluting to 1 litre. A 0.1% working solution was prepared by dilution and stored in the dark.

Assays were carried out in 25 ml. flasks containing 5 ml. buffer and 2 ml. of 0.1% amylose solution. Appropriate dilutions of amylase-containing solutions were added to substrate-buffer mixture, pre-warmed to 35°C, and the assay incubated at 35°C for 15 minutes. The contents of each assay flask was then diluted quickly to approximately 80 ml. with distilled water and 4 ml. of the iodine-potassium-iodide solution added. Blanks were carried out in which the enzyme was added immediately prior to addition of iodine-potassium-iodide solution. Volumes were made up to 100 ml. in measuring cylinders and optical densities measured in an EEL colorimeter (608 filter) against a water blank. The percentage hydrolysis of the amylose can then be calculated as follows:-

$$\% \text{ hydrolysis} = \frac{O.D._{blank} - O.D._{test}}{O.D._{blank}} \times 100$$

The accuracy and the reproducibility of the assay fall off rapidly if hydrolysis exceeds 50% and hence enzyme dilution has to be made by trial and error until a suitable one is found.

Definition of amylase units

Many different units of amylase activity have been introduced by workers using the various techniques mentioned above. The unit used here is based most closely on the Street-Close unit for serum amylase levels determined by the above iodine complex method [Street and Close, 1958] and is defined as follows:-

1 amylase unit is that amount of amylase required to cause a 10% hydrolysis in 15 minutes at 35°C under the experimental conditions described above.

The International Unit of enzyme activity (1 μmole of substrate converted per minute) is not readily applicable for use in this assay.

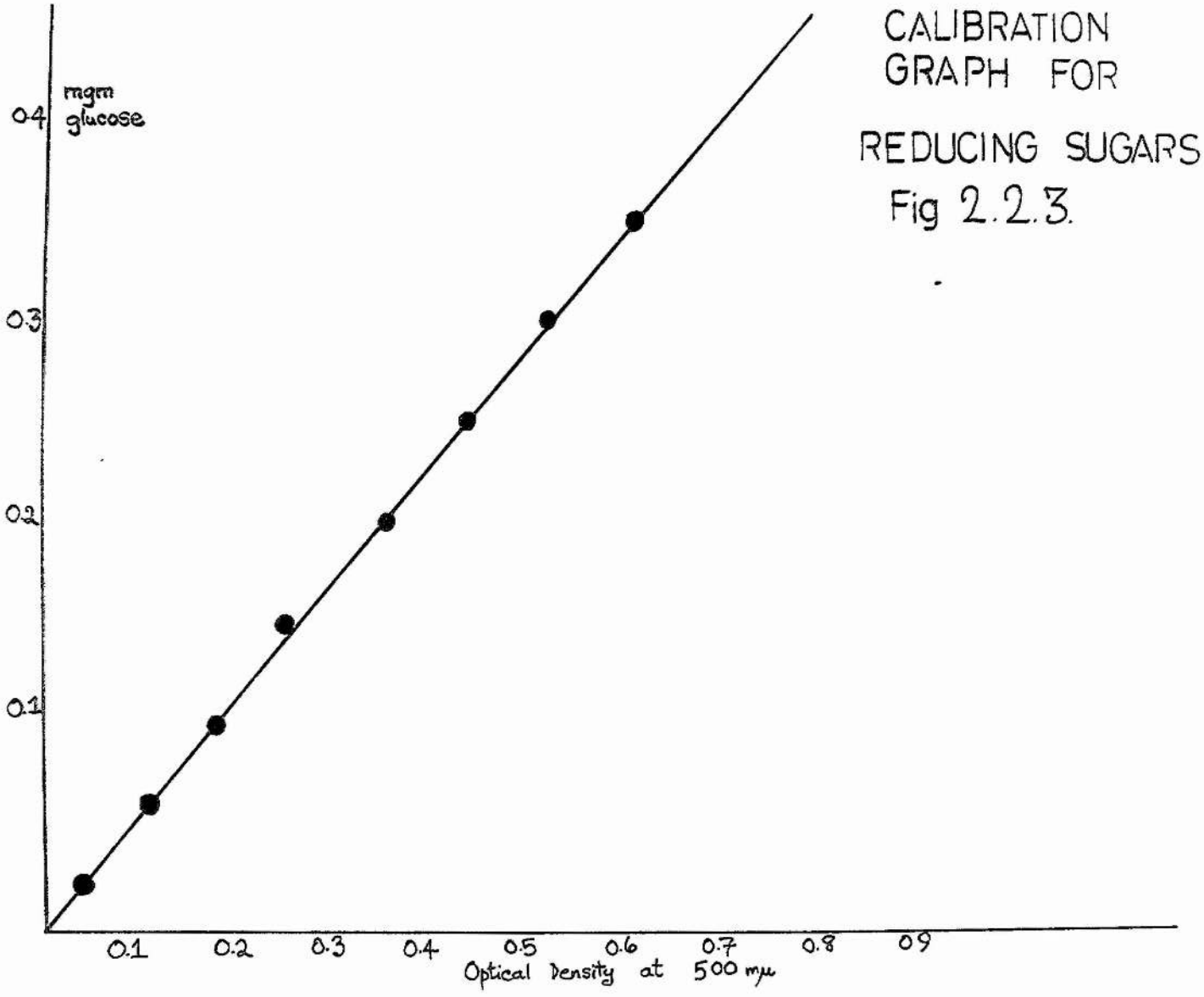
2.2.2. Estimation of protein

Protein was estimated by the turbidimetric method of Bucher (1947) adapted by Kornberg (1950). The procedure is as follows:-

To the protein sample containing not more than 0.8 mg. of protein in 2.6 ml. is added 0.5 ml. of 0.6M ammonium sulphate and the extinction at 340 mμ is read on a Unicam SP500 spectrophotometer with a cuvette of 1 cm. light path. 0.1 ml. of 50% (w/v) trichloroacetic acid is then added, with mixing, and the extinction read again after 2 minutes. With most proteins, including those of microbial extracts, the increase in extinction is directly proportional tb the amount of protein present. This increase was approximately equal to 1.0 per mg. protein present, and since the choice of a standard protein is arbitrary, this value was used, for speed and convenience, throughout.

2.2.3. Determination of reducing sugars

Reducing sugar was estimated by Nelson's colorimetric modification of Somogyi's method [Hodge and Hofreiter,1962] using Somogyi's (1952) low-alkalinity copper reagent and Nelson's (1944) arsenomolybdate reagent. The procedure is as follows:-



CALIBRATION GRAPH FOR REDUCING SUGARS

Fig 2.2.3.

Reagents

(i) Low-alkalinity copper reagent:- Sodium potassium tartrate (12 g.) and anhydrous sodium carbonate (24 g.) are dissolved in about 250 ml. water. A solution of 4.0 g. of cupric sulphate pentahydrate in water is added with stirring, followed by 16 g. of sodium hydrogen carbonate. A solution of 180 g. of anhydrous sodium sulphate in 500 ml. of water is boiled to expel air; then the two solutions are combined and diluted to 1 litre.

(ii) Arsenomolybdate reagent:- To 25 g. of ammonium molybdate in 450 ml. of water is added 21 ml. of 96% sulphuric acid, followed by 3 g. of disodium hydrogen arsenate heptahydrate dissolved in 25 ml. of water. The mixed solution is incubated for 24 hours at 37°C and stored protected from light in a brown bottle.

Assay: To 1 - 5 ml. of sugar solution containing not more than 0.6 mg. of glucose or its equivalent, an equal volume of low-alkalinity copper reagent is added. Samples and blanks are heated for 10 minutes in a vigorously boiling water-bath and then cooled. 2Ml. of arsenomolybdate reagent is then added with caution and the tubes are carefully mixed with glass rods to dissolve the cuprous oxide. The solutions are now diluted to 20 ml. in volumetric flasks and allowed to

stand for 15 minutes after which optical densities are measured at 500 mμ on a Unicam SP600 spectrophotometer. [N.B. Measurement at 500 mμ, although not the absorption maximum of the blue-green arsenomolybdous acid formed in this reaction, gives in spite of reduced sensitivity, greater accuracy over a wider range.]

A calibration curve was established using a standard reagent grade glucose solution. (See Fig. 2.2.3)

2.2.4. Determination of total carbohydrate

Total carbohydrate was determined by the phenol-sulphuric acid method [Dubois et al., 1956]. This method is widely applicable to all classes of carbohydrate and is rapid, accurate and sensitive. Determinations were carried out in duplicate and care was taken to avoid any contamination from filter papers, cellulosic tissues etc.

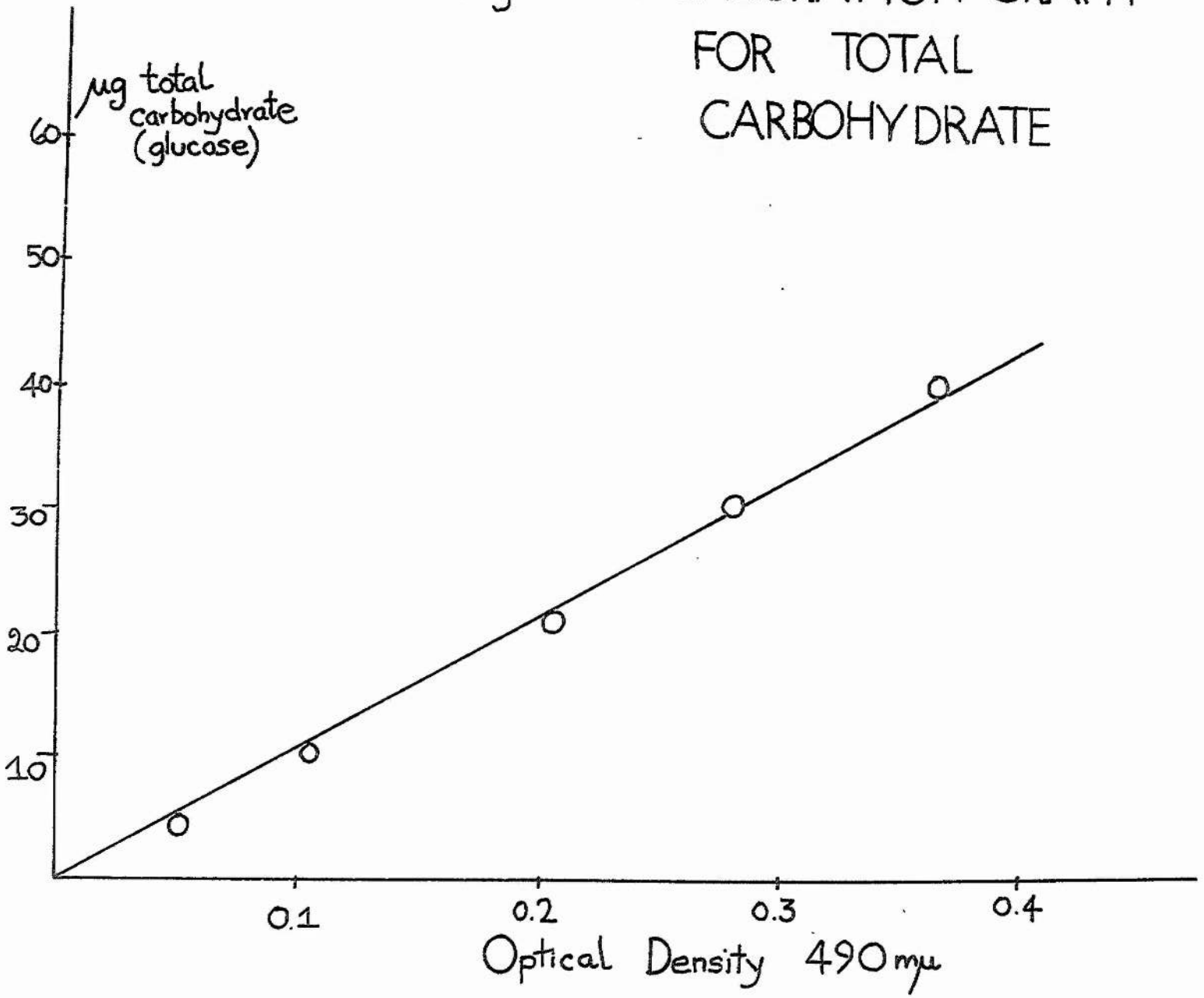
Reagents

(i) 5% aqueous phenol was prepared from reagent grade phenol and was stable over long periods.

(ii) 96% sulphuric acid was reagent grade

Assay: To 1 ml. of aqueous solution containing not more than 0.07 mg. of carbohydrate is added 1 ml. of phenol reagent and the tube mixed. From a burette with a sawn-off tip is added rapidly 5 ml. of sulphuric acid reagent

Fig 2.2.4. CALIBRATION GRAPH FOR TOTAL CARBOHYDRATE



in a steady stream directed on to the surface of the liquid in the tube. Considerable heat is generated and good mixing is achieved. It is important to perform exactly the same mixing procedure throughout. The tubes are re-shaken after 10 minutes and then again after a further 20 minutes at room temperature. Optical densities are then measured at 490 mμ (for hexoses) against blanks in which water replaces the sugar solution.

Calibration curves were prepared using glucose and potato amylose and were found to differ only slightly at low concentrations. (See Fig. 2.2.4)

2.2.5. Paper chromatography

Whatman 3MM chromatography paper was used throughout this work, normally stapled in the form of a cylinder and employed in the ascending technique. The solvent system used was n-butanol: pyridine : water :: 3:2:1.5. As the R_f values of members of the maltosaccharide series differ only fractionally, the technique of multi-ascent chromatography was used, this involving the development of the chromatogram several times in the same direction with the same solvent system, drying between each run. Generally adequate separation between components was obtained after 3-4 ascents.

Two-dimensional paper chromatography was also employed in the investigations of enzyme action on individual maltosaccharides. This involves separation of the sugars in a partial acid hydrolysate of amylose in one direction, spraying of the separated sugars *in situ* with an aqueous solution of enzyme and then development in the second direction. Chromatograms were visualised after drying by treatment with alkaline silver nitrate [Trevelyan, Proctor and Harrison *et al.*, 1950].

Reagents:

(i) Silver nitrate reagent: Add 0.1 ml. of saturated aqueous silver nitrate to 20 ml. of acetone. Add water dropwise with stirring until the silver nitrate just dissolves. Store in a dark bottle.

(ii) NaOH reagent: Dissolve 10 g. of sodium hydroxide in 10 ml. of water and make up to 500 ml. with absolute ethanol.

Chromatograms are dipped in the silver nitrate reagent, dried, then sprayed with the NaOH reagent. Maltosaccharides appear as dark brown to light brown spots against a lighter background. The background is cleared by washing in 6N ammonia briefly and then in running tap water for 30 minutes.

2.2.6. Preparation of amylose from potato starch

Amylose for the preparation of maltosaccharides was prepared (Street, 1963) from potato starch (BDH Ltd.) which had been exhaustively extracted with methanol to remove lipid materials. A 30 g. batch of potato starch was made into a cream with 150 ml. of water and poured into 1.5 litres of boiling 2% NaCl solution. The solution was stirred until homogeneous and then filtered hot through muslin. To the filtrate was added 4.5 g. of powdered thymol and the mixture was mechanically stirred for 48 hours. The amylose precipitates as a thymol-amylose complex and the supernatant which is mainly amylopectin is discarded. The precipitate is washed six times with thymol-saturated water and then four times with ethanol. The amylose is then dried on a glass plate. The yield is about 3 g.. of amylose from 30 g. of potato starch.

2.2.7. Preparation of a partial-acid hydrolysate of amylose

Controlled acid hydrolysis of amylose can be used to produce a mixture of all the maltosaccharides from glucose to maltooctaose and possibly higher ones.

0.4 g. of amylose was rapidly dispersed in 100 ml. 0.1M HCl at 100°C. and maintained at this temperature on a water-bath for 1 hour. This treatment was found to give approximately 55% conversion to glucose in terms of reducing equivalents. The resultant maltosaccharide solution was cooled rapidly and neutralised by the addition of sufficient Amberlite IR 45 anion exchange resin. The resin was filtered off and washed with distilled water to minimise the loss of carbohydrate by non-specific adsorption to resin. The maltosaccharide solution was reduced to dryness under reduced pressure in a rotary evaporator, and taken up in a small volume of water to form a syrup for chromatography.

2.2.8 Preparation of maltosaccharides by multi-ascent chromatography.

Maltosaccharide syrup was applied as a strip along the lower edge of a sheet of Whatman 3MM paper and four ascents were carried out in the solvent system described previously. A 2-inch strip was cut from one edge of the chromatogram and stained with alkaline silver nitrate. Using this as a guide, the unstained remainder of the chromatogram was cut into zones containing the individual

oligosaccharides which were subsequently eluted into warm water in test-tubes. Such aqueous solutions were stored in the frozen state. G4 and G5 were prepared in this manner.

2.2.9. Purification of maltose

The purest grades of maltose commercially available contain small amounts of glucose and higher oligosaccharides by virtue of their mode of manufacture. Maltose can be purified by crystallization but this is a difficult procedure and purification is still not complete. The pure sugar can be prepared by deacetylation of β-maltose octa-acetate which is easily purified by recrystallization. This was carried out as described by Wolfrom and Thompson (1962), with the following difference. The maltose was not crystallized, but stored as a methanol solution at -15°C. Paper chromatography revealed it free of other sugar contaminants.

2.2.10. Preparation of maltotriose

Maltotriose was prepared, in rather larger quantities than was possible by hydrolysis and paper chromatographic separation techniques, from salivary amylase digests of

amylose. This was carried out by the method described by Pazur (1962).

2.2.11. Preparation of glycogen.

Glycogen was extracted from the edible mussel, Mytilus edulis. Protein was removed by precipitation as insoluble picric acid salts; the glycogen was purified by repeated precipitation with ethanol followed by ion-exchange treatment and was finally dried over phosphorous pentoxide.

2.3. Preparation of CM-cellulose-amylase

The insoluble derivative CM-cellulose-α-amylase was prepared by chemically coupling purified α-amylase to CM-cellulose^{-32} according to the procedure of Mitz and Summaria (1961). The carboxymethyl side chain of the CM-cellulose has to be modified prior to coupling and the actual coupling occurs between CM-cellulose acid azide and the enzyme. The steps involved are as follows:

$$R\text{-}O\text{-}CH_2\text{-}COOH \xrightarrow{HCl/CH_3OH} R\text{-}O\text{-}CH_2.COOCH_3 \xrightarrow{NH_2.NH_2/CH_3.OH}$$

CM-cellulose — CM-cellulose methyl ester

$$R\text{-}O\text{-}CH_2.CO.NH.NH_2 \xrightarrow{NaNO_2/HCl} R\text{-}O\text{-}CH_2\text{-}CO.N_3$$

$$R\text{-}O\text{-}CH_2\text{-}CO.N_3 + H_2N\text{-}Enzyme \xrightarrow{pH\ 8.7} R\text{-}O\text{-}CH_2.CO.NH.Enz.$$

amylase — CM-cellulose-amylase

The CM-cellulose acid hydrazide was prepared in batches and after drying over anhydrous calcium chloride in a vacuum desiccator was very stable and could be stored at room temperature for several months. The CM-cellulose acid azide was relatively unstable and was prepared immediately prior to coupling to the enzyme. 3.5 gm. of CM-cellulose acid hydrazide was treated with 0.2% w/v sodium nitrite in 0.6 M HCl for 20 minutes at 0°C. The resultant CM-cellulose acid azide was washed twice with dioxan at 12°C. and dried on a filter funnel. Immediately 50 ml. of water at 1°C. was added and the suspension titrated to pH 8.7 with alkali. 450 Ml. of pyrophosphate buffer, pH 8.7, and 450 ml. of purified, dialysed amylase solution (approximately 5 mg./ml. protein), both ice-cold, were added and the mixture stirred magnetically for 60 minutes with cooling in an ice/salt bath. The CM-cellulose-amylase

was collected by centrifugation at 4°C. and successively washed at 4°C. with M NaCl, 0.5M $NaHCO_3$ and M NaCl. The insoluble enzyme was then stored as a water suspension at 4°C.

2.3.1. Determination of chemically-bound protein in CM-cellulose amylase preparations

The protein content of insoluble CM-cellulose amylase was determined by hydrolysis of samples with 6N HCl at 120°C. for 40 hours followed by estimation of the liberated amino-acids by the method of Cocking and Yemm (1954). The protein content was calculated from a calibration curve produced by hydrolysing known amounts of the same amylase as was used in the preparation of the insoluble derivative.

2.4.

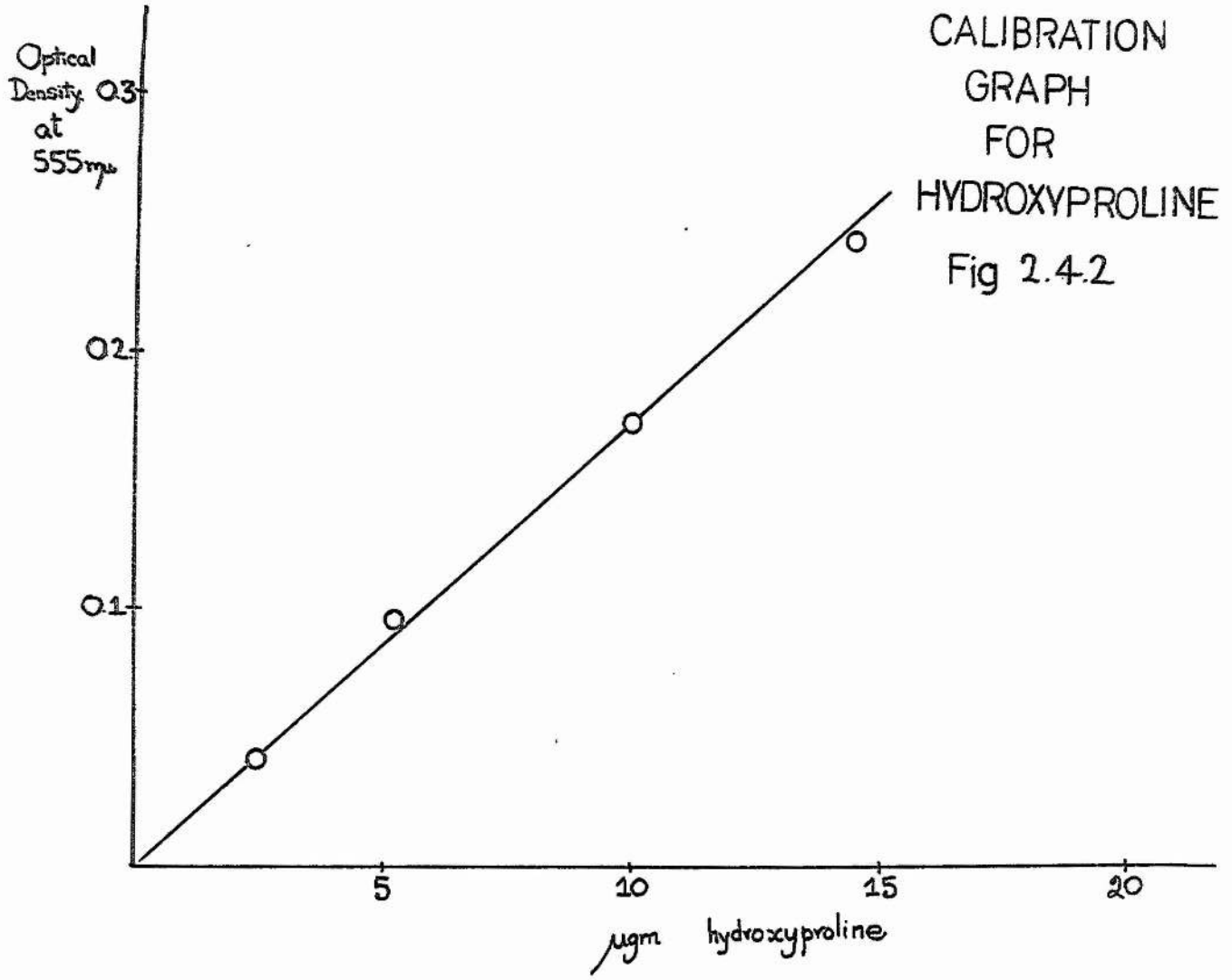
2.4.1. Preparation of red deer tendon collagen

Achilles tendons were dissected from legs of red deer and cleaned of non-collagenous tissue as far as possible. They were then cut into small pieces and minced in a Waring Blendor in 0.2M disodium hydrogen phosphate. The insoluble tissue was washed once in this solution and then with distilled water and partially freeze-dried. Partially

freeze-dried tissue was frozen in liquid nitrogen and could then be pulverised in a mortar. The pulverised tissue was thoroughly freeze-dried and stored in an air-tight container at room temperature.

2.4.2. Assay of 'collagen-liberating' activity of bacterial preparations

Bacterial enzyme preparations were allowed to act on suspensions of 50 mg. of freeze-dried tendon tissue at room temperature for periods of 24 hours. The washed tissue was then extracted with 0.2M acetic acid for 24 hours. Aliquots of the extract were hydrolysed with 6N HCl for 18 hours and the neutralised hydrolysates assayed for hydroxyproline by the method of Stegemann (1958). (See Fig. 2.4.2)



CALIBRATION GRAPH FOR HYDROXYPROLINE

Fig 2.4.2

RESULTS

R E S U L T S

3.1

3.1.1. Preliminary experiments with B. subtilis

Preliminary experiments with B. subtilis 3610 on nutrient broth media showed that growth was rapid during a 12 hr. experimental period under conditions of maximal aeration at a temperature of 35°C. At the end of this period supernatant amylase levels were normally in the range 10-15 units/ml. of supernatant. Other strains of B. subtilis produced less amylase under these conditions and were not studied further.

Experiments with basal media supplemented with 4% soluble starch showed greatly increased levels of supernatant amylase. The normal range was 25-30 units/ml. after 24 hours, rising to 50 units/ml. in 3 days. After 5 days, levels were found to fall. In all the above experiments, cells were removed by centrifugation at 5000 g. for 20 minutes and the resultant supernatant assayed after appropriate dilution.

Table 3.1.1.

Age of Culture DAYS	Supernatant amylase level UNITS/ML
1	25-30
3	50-55
5	45-50
8	30-35
10	10-15

3.1.2. Growth experiments with B. subtilis and the effect of various carbon supplements on characterisitics of amylase production

Experiments were carried out to determine the effect of various carbon sources as inducers of amylase formation in growing cells. The basal medium was supplemented with 0.1% of the carbon source under investigation and the inoculum was a fresh 12-hr. culture grown in the same medium, added to give an initial optical density of 0.075 - 0.1 at 610 mμ. Over the experimental period of 5 - 6 hrs. the following parameters were followed:-

1) Optical density of the culture
2) Supernatant amylase level
3) Total amylase level as determined in a 10 min. sonicate of the culture

With glucose and sucrose as carbon supplements it was found that intracellular levels of amylase tended to rise rapidly until the start of the logarithmic phase when they levelled off. Around mid-logarithmic phase the extracellular amylase levels started to rise and continued to do so until the end of the experimental period (See figures 3.1.2a and 3.1.2b). The intracellular amylase level was found to be 3.5 units/mg. dry bacteria with glucose

Figures 3.1.2 a,b,c,d

Itshould be carefully noted that, in the above figures, as intracellular amylase levels are plotted, for convenience, as activities/ml and not as activities/cell or mg. ,cellular nitrogen, a levelling off of intracellular amylase curves in fact indicates a fall in intracellular amylase levels in terms of activity/cell.

Fig 3.1.2 (a) AMYLASE PRODUCTION WITH GLUCOSE AS CARBON SUPPLEMENT

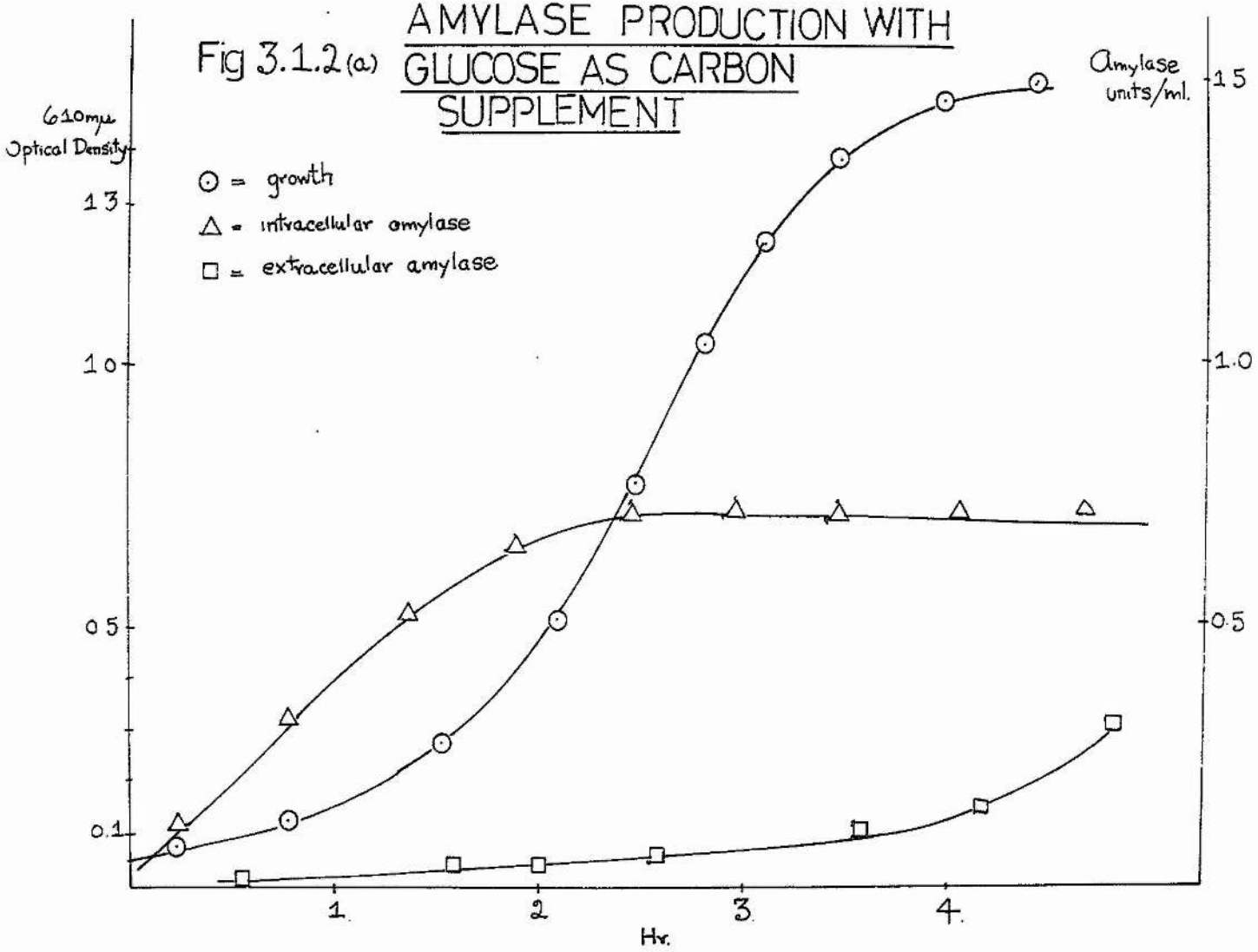
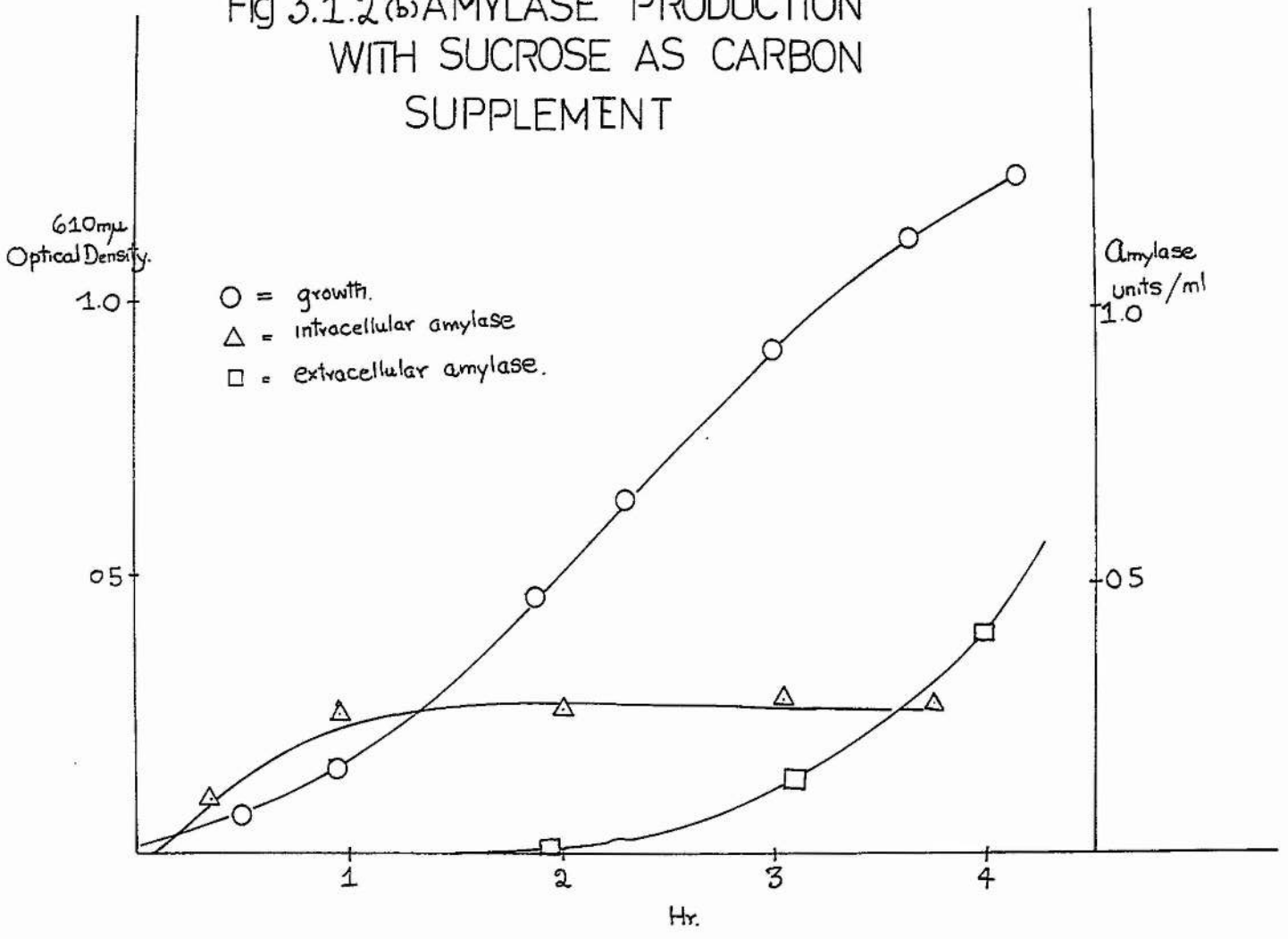


Fig 3.1.2 (b) AMYLASE PRODUCTION WITH SUCROSE AS CARBON SUPPLEMENT



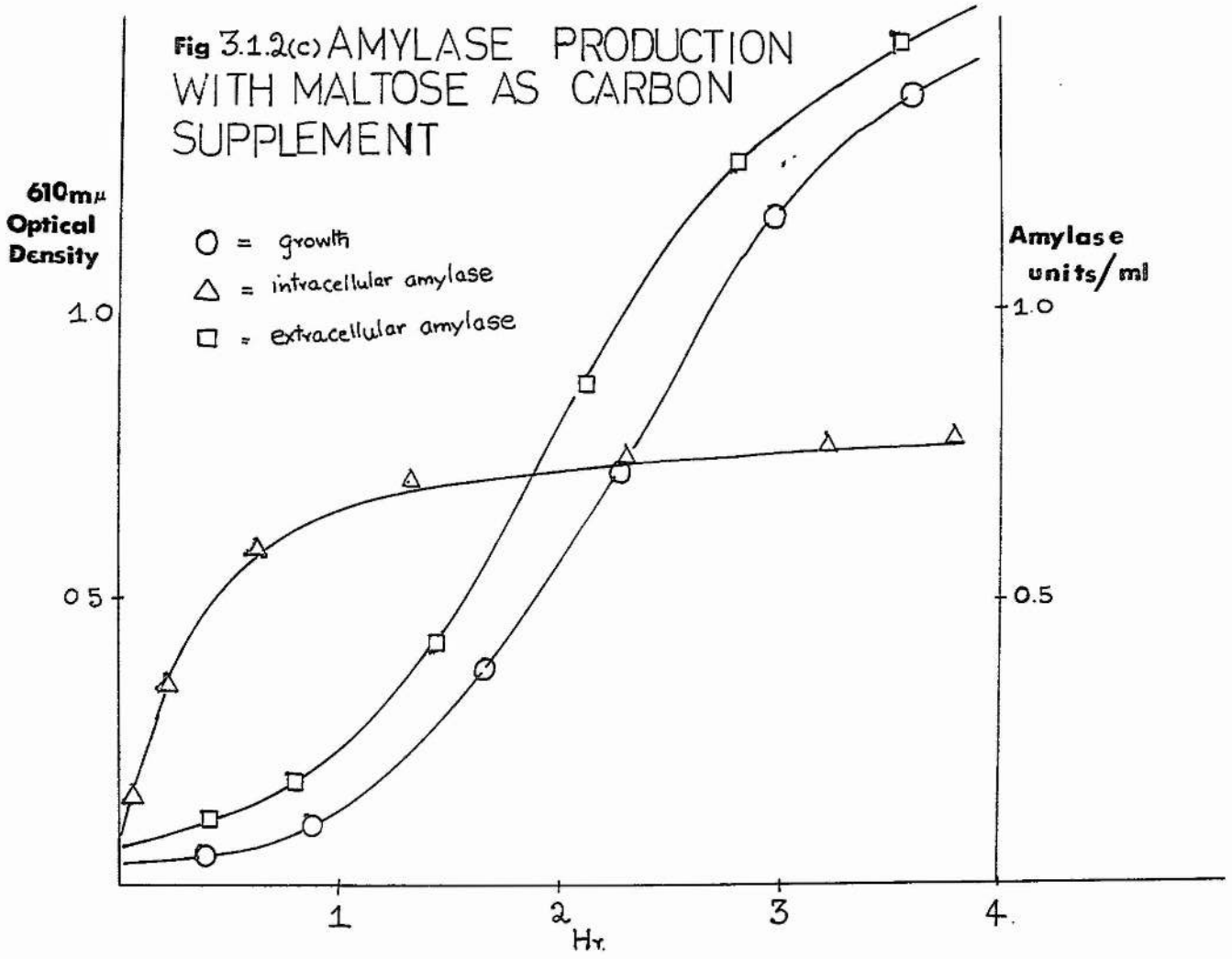


Fig 3.1.2(c) AMYLASE PRODUCTION WITH MALTOSE AS CARBON SUPPLEMENT
610mμ Optical Density
Amylase units/ml
O = growth
△ = intracellular amylase
□ = extracellular amylase
1.0
0.5
1.0
0.5
1
2
3
4
Hr.

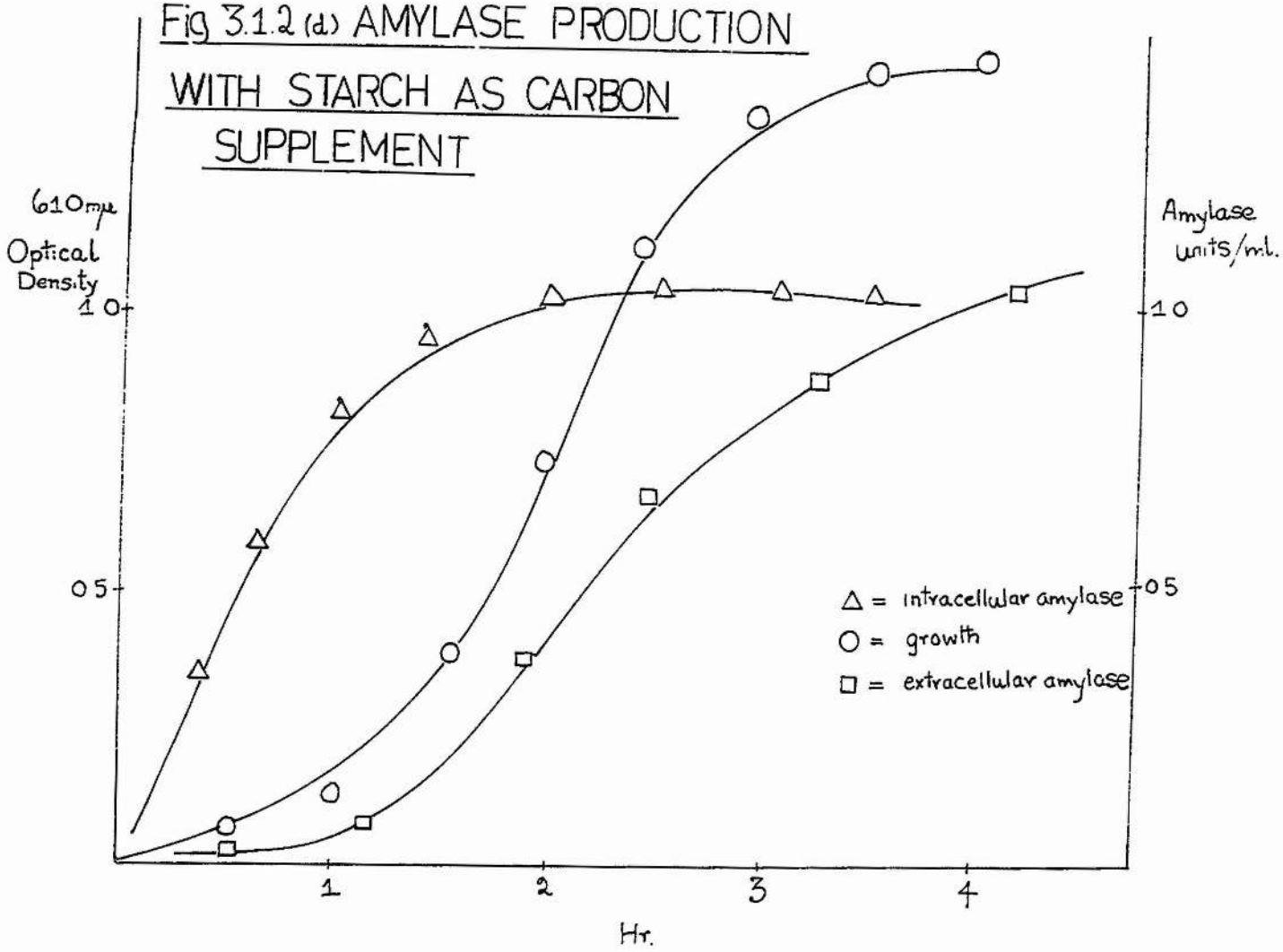


Fig 3.1.2 (d) AMYLASE PRODUCTION WITH STARCH AS CARBON SUPPLEMENT
610mμ
Optical Density
1 0
0 5
Amylase units/m.l.
1 0
0 5
△ = intracellular amylase
○ = growth
□ = extracellular amylase
1
2
3
4
Hr.

supplementation and 2.3 units/mg. dry bacteria with sucrose supplementation at the start of the logarithmic phase.

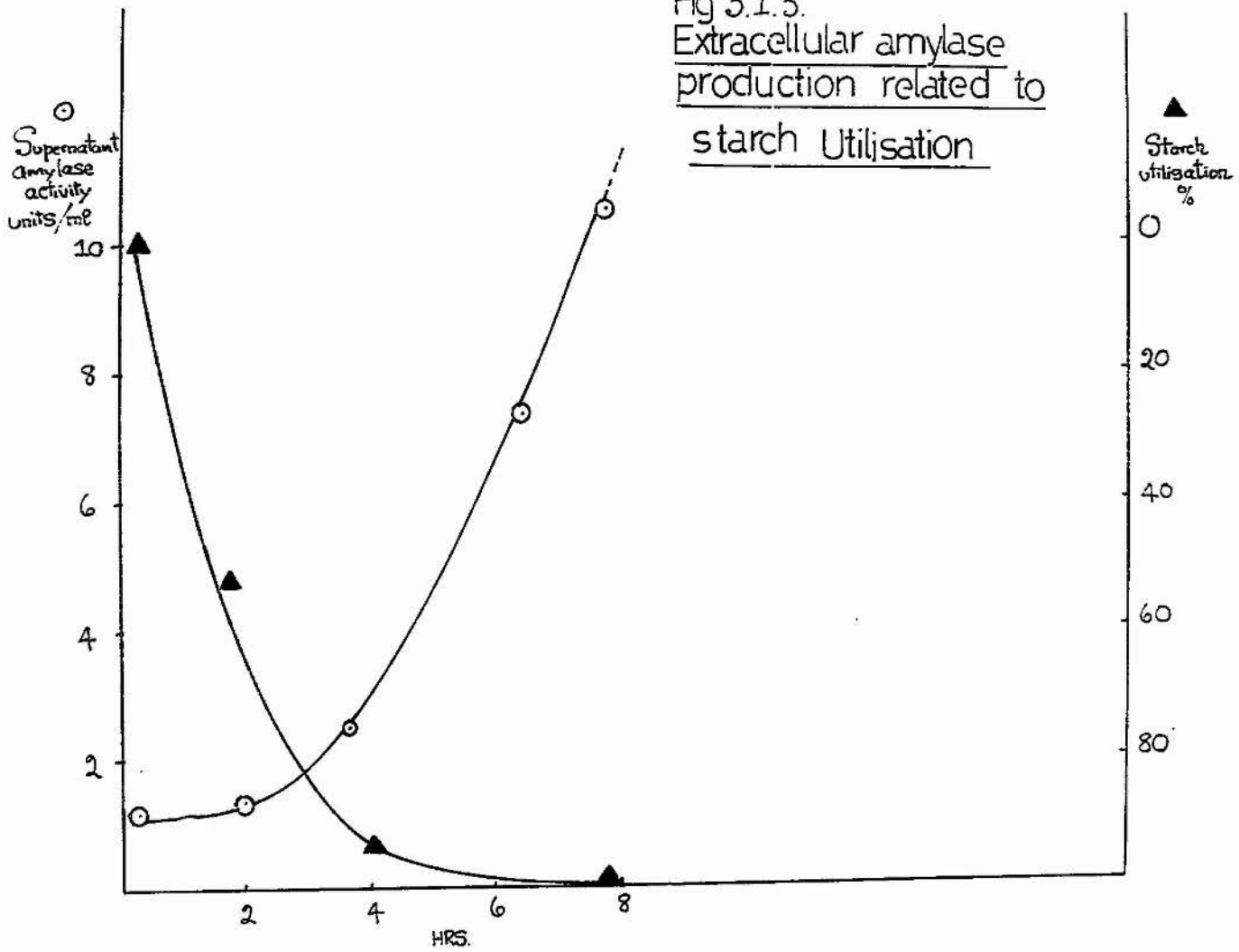
With maltose as the carbon supplement, the initial intracellular level of amylase again rose rapidly during the ~~logarithmic~~ lag phase and levelled off, while extracellular amylase tended to increase with growth, suggesting that enzyme liberation was taking place concomitantly with synthesis. The ~~=====~~ intracellular enzyme level was some 10 units/mg. dry bacteria at the start of the logarithmic phase (see fig. 3.1.2c)

With starch as carbon supplement, the characteristics of amylase formation were again different. The intracellular enzyme level was found to increase sharply during the lag phase and then to fall off in ~~=====~~ logarithmic phase as extracellular levels rose slowly. ~~At mid-logarithmic phase, intracellular levels started to increase again and paralleled growth (see fig. 3.1.2d).~~ The intracellular level at the start of the logarithmic phase was 12 units/mg. dry bacteria.

3.1.3. Characteristics of amylase production over a longer period with 1% starch as the carbon source

In a culture with 1% starch as the carbon supplement, extracellular amylase levels were followed over a period

Fig 3.1.3.
Extracellular amylase production related to starch Utilisation



of 3 days. The culture was aerated maximally for 18 hours and then aeration was discontinued during the remainder of 3 days at 35°C. Figure (3.1.3) shows the increase in extracellular amylase in relation to the remaining starch concentration for the earlier part of the experimental period. It is of interest to note that extracellular amylase levels do not apparently increase appreciably until the starch concentration has fallen to some 10% of its initial level. From this time extracellular amylase levels rise rapidly to a value of 30 units/ml. after 18 hours. After a further period of incubation at 35°C. without aeration, an enzyme level of 54 units/ml. existed at 72 hours. Further incubation did not increase this level.

3.1.3(a) Characteristics of amylase production by washed cells of B. subtilis in the presence of different carbon supplements

Bacteria were grown in the basal medium supplemented with 0.1% glucose for 12 hours, at which time the bacteria were in the stationary phase. They were harvested by centrifugation at 5000 g/ for 20 minutes, washed once in 0.1M phosphate buffer, pH 7.0, and resuspended in the same

buffer to an optical density of 0.4 - 0.6 in the presence of the particular carbon supplement. Figure (3.1.3a) illustrates the amylase production curves and Figure (3.1.3b) illustrates the extent to which lysis took place in these experiments.

Table 3.1.3 (a)

Effect of carbon supplements, at 0.1% concentrations, on the liberation of amylase from washed cells of B. subtilis

	Units amylase liberated/mg. dry bacteria/hr.
Distilled water	0.11
Phosphate Buffer	0.3
Amylose	0.5
Amylopectin	0.8
Maltotriose	1.1

N.B (1) As the cells were lysing slowly (see fig. 3.1.3b) during the experimental period, the dry weight of bacteria parameter is a calculated mean value for each experiment.

(2) As enzyme may be liberated during the cell washing procedure the experimental zero ~~xx~~ time on Fig 3.1.3 a is arbitrary

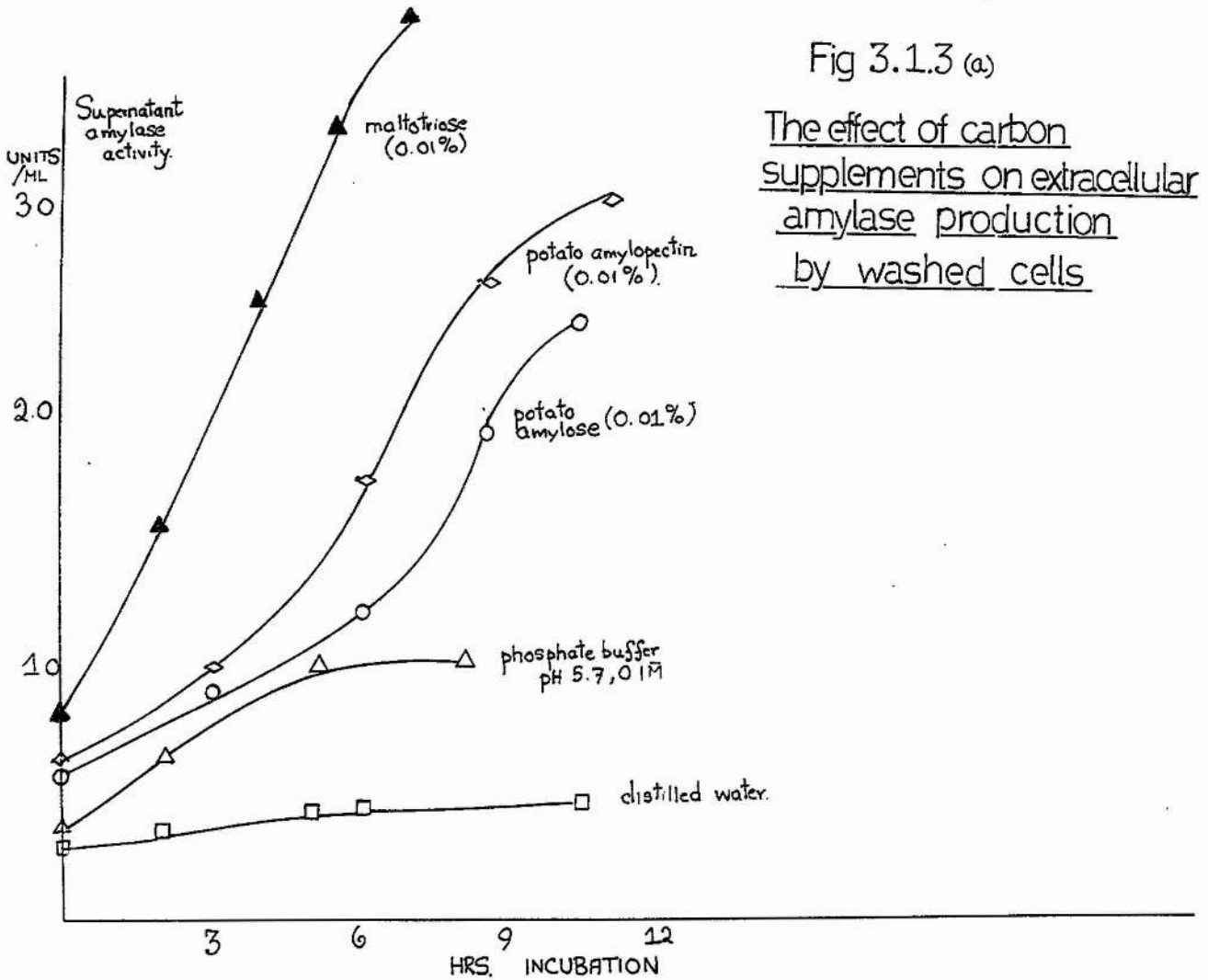


Fig 3.1.3 (a)

The effect of carbon supplements on extracellular amylase production by washed cells

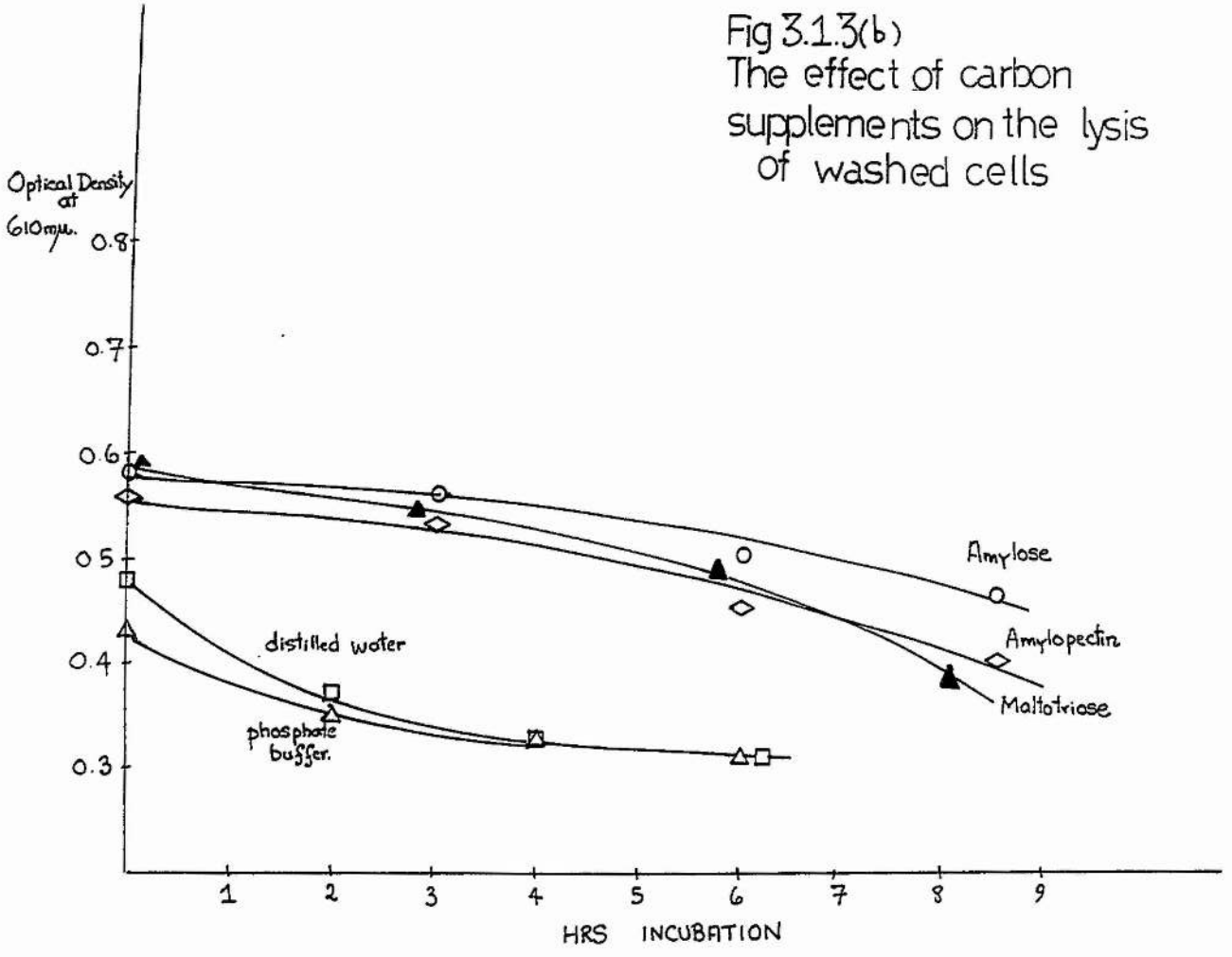


Fig 3.1.3(b)
The effect of carbon supplements on the lysis of washed cells

3.1.4. The effect of the presence of an exogenous nitrogen supply on the production of amylase by washed cells in the presence and absence of maltotriose

The ability of washed cells to produce amylase in the presence of both maltotriose, which was the most effective of the carbon sources tested, and 0.1% casein hydrolysate, a source of amino acids, was investigated. Figure (3.1.4) illustrates the progress of amylase liberation by cells in the presence of casein hydrolysate with maltotriose and in the presence of casein hydrolysate alone. The rate of enzyme liberation was much faster in the presence of maltotriose. In its absence, a considerable lag period was evident before enzyme liberation commenced.

Table 3.1.4

The effect of a nitrogen source on amylase liberation in washed cells in the presence and absence of maltotriose.

Supplement	Units amylase liberated/ mg. dry bacteria/hr.
	Average over 6-hr period [0 to 6 hrs]
Maltotriose + casein hydrolysate	10.0
Casein hydrolysate	1.2

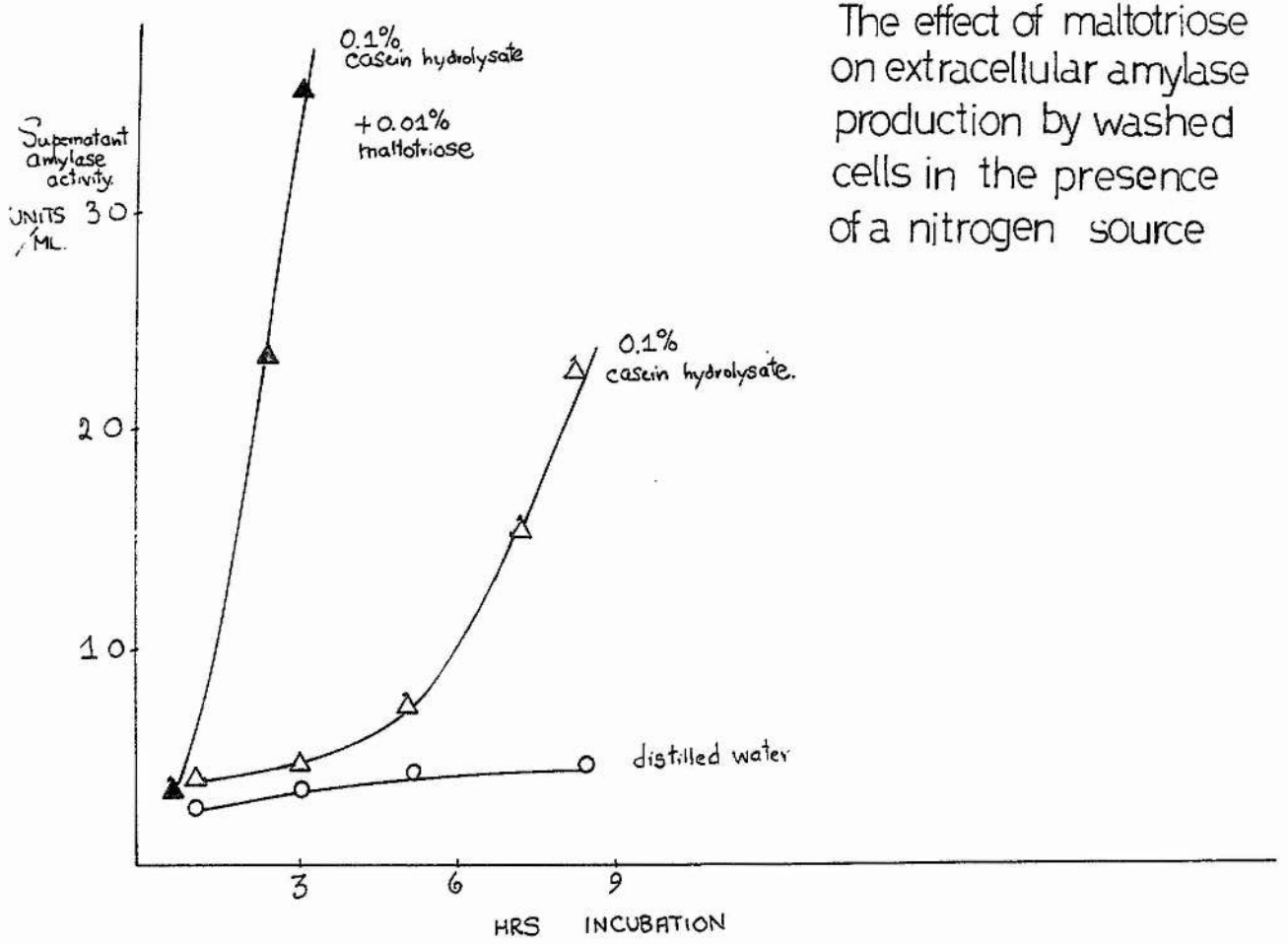


Fig 3.1.4.
The effect of maltotriose on extracellular amylase production by washed cells in the presence of a nitrogen source

3.2 Studies of amylase production by B. subtilis under continuous culture conditions

Using the simple continuous culture apparatus constructed from standard laboratory equipment, experiments were carried out to investigate the characteristics of amylase production during the logarithmic phase of growth. Continuous culture techniques permit the study of bacteria of which a high proportion are in the logarithmic phase of growth throughout the experimental period, and the interpretation of any observations is not complicated by the ever-changing biochemical and physiological environment of the organisms as is always the case with growth under batch conditions.

As the apparatus was home-made, preliminary blank runs were carried out to ensure that it could be run for considerable periods without risk of contamination. With a 2% glucose/tryptone medium and a dilution rate of 0.5 $hr.^{-1}$, the apparatus remained sterile over the testing period of 5 days. Experimental runs were normally of slightly shorter duration (3-4 days).

Experimental runs were carried out at 4 different concentrations of starch as carbon and energy supplement

to the basal medium and the results described here are each the average of 2 runs. After sterilisation and assembly of the apparatus, the reactor vessel was inoculated with 10 ml. of a fresh 18-hr. culture of B. subtilis and allowed to grow under batch conditions for 2 hours, with aeration and stirring, at the end of which time the media-in and media-out pumps were started. Growth under continuous culture conditions then proceeded for 30-40 hr. when a steady state of growth was evident from optical density readings. During the experimental period samples of the culture were withdrawn from the reactor at hourly intervals for 4 hours and amylase assays carried out on the supernatant. The steady state concentration of starch was also determined.

Table 3.2.1

The effect of starch concentration on the liberation of amylase during continuous culture

Temperature 35°C
Dilution rate 0.5 $hr.^{-1}$

Starch concentration of medium (%)	Starch concentration in reactor (%)	Optical Density	Supernatant Amylase (units/ml
0.1	0.017	0.21	0.5
0.2	0.02	0.51	0.1
0.3	0.12	1.5	0.1
0.7	0.4	1.58	0.1

From the original starch concentration and the steady state level in the reactor vessel it is possible to calculate the yield (i.e. the weight of bacteria produced per given weight of starch metabolised),

Table 3.2.2

The effect of starch concentration on the yield of bacteria

Starch concentration of medium (%)	Yield (gm./gm. starch metabolised)
0.1	0.12
0.2	0.14
0.3	0.6

3.3 Studies on the purification of amylase from B. subtilis

The starting material for purification experiments was a cell-free culture supernatant obtained by centrifugation of batch cultures at 6000 g. for 20 minutes. Filtration of cultures using filter aid (Celite) was tried in earlier experiments but amylase losses were substantial due to non-specific protein absorption by the filter aid. Cultures were always allowed to staud at 35°C. for 2 days after aeration was discontinued (normally 18 hours) in order to obtain maximal yields of extracellular amylase. Cellular material examined at this stage contained considerable quantities of bound amylase. Attempts to solubilise this amylase by sonication resulted in little soluble amylase after 15 min., while the original cellular material retained considerable activity. Efforts to solubilise the enzyme with the detergent sodium lauryl sulphate met with similar lack of success. It is therefore assumed that cellular amylase is tightly bound in this bacterium.

3.3.1 Ammonium sulphate fractionation of amylase

The results indicated below are typical ones obtained in the fractionation of B. subtilis amylase with ammonium sulphate.

Stage	Volume (ml.)	Total protein (mg.)	Total enz activity (units)	Spec. activity (units/mg.)	Yield % (Total enzyme activity)
Crude culture filtrate	1400	180	54,000	300	(100)
0-100% Ammonium sulphate fraction	150	80	38,000	476	70
Ammonium sulphate fractions					
0 - 20%	10	5	200	33	0.3
20 - 40%	10	29	1,000	35	1.8
40 - 60%	17	15	30,000	1900	55
60 -100%	12	10.5	310	30	0.4

3.3.2. Fractionation on DEAE-cellulose

3Ml. of the 40-60% ammonium sulphate fraction were applied to the top of a 30 cm. column of DEAE-cellulose equilibrated with 0.01M calcium acetate buffer, pH 6.1.

Elution was attempted with the same buffer with a stepwise increase in ionic strength through 0.01M, 0.025M and 0.05M. The protein was found to be loosely bound and came off with the solvent front. Further attempts at pH 6.8, at which pH amylase should be more negatively charged and should bind tighter, delayed the appearance of protein off the column until elution with 0.05M buffer. However there was no distinct peak of amylase activity and no increase in specific activity was found. In view of the unlikelihood of obtaining a substantial increase in specific activity by this method no further attempts were made to improve the fractionation.

3.3.3. Specific absorption of amylase to glycogen and starch.

Preliminary experiments were carried out with a commercial amylase from B. subtilis (Cambrian Chemical Co. Ltd.) (specific activity, approximately 13,000 units/mg. protein). The amylase was dissolved in 50% ethanol-50% phosphate buffer solution at pH 5.7 at 0°C. Ice-cold glycogen solution was added with constant magnetic stirring which was continued for 5 minutes after

precipitation of the amylase-glycogen complex was complete. The insoluble complex was collected by centrifugation at 5000 g. for 20 minutes and the supernatant checked for residual amylase activity. Starch-amylase complex was prepared in an identical manner.

Figure (3.3.3.) shows the amount of amylase adsorbed by different amounts of glycogen or soluble starch in a reaction volume of 25 ml. containing 18,000 units of amylase. Soluble starch appears to be slightly less efficient as an adsorbent under these conditions. The calculated ratios of enzyme to substrate are:-

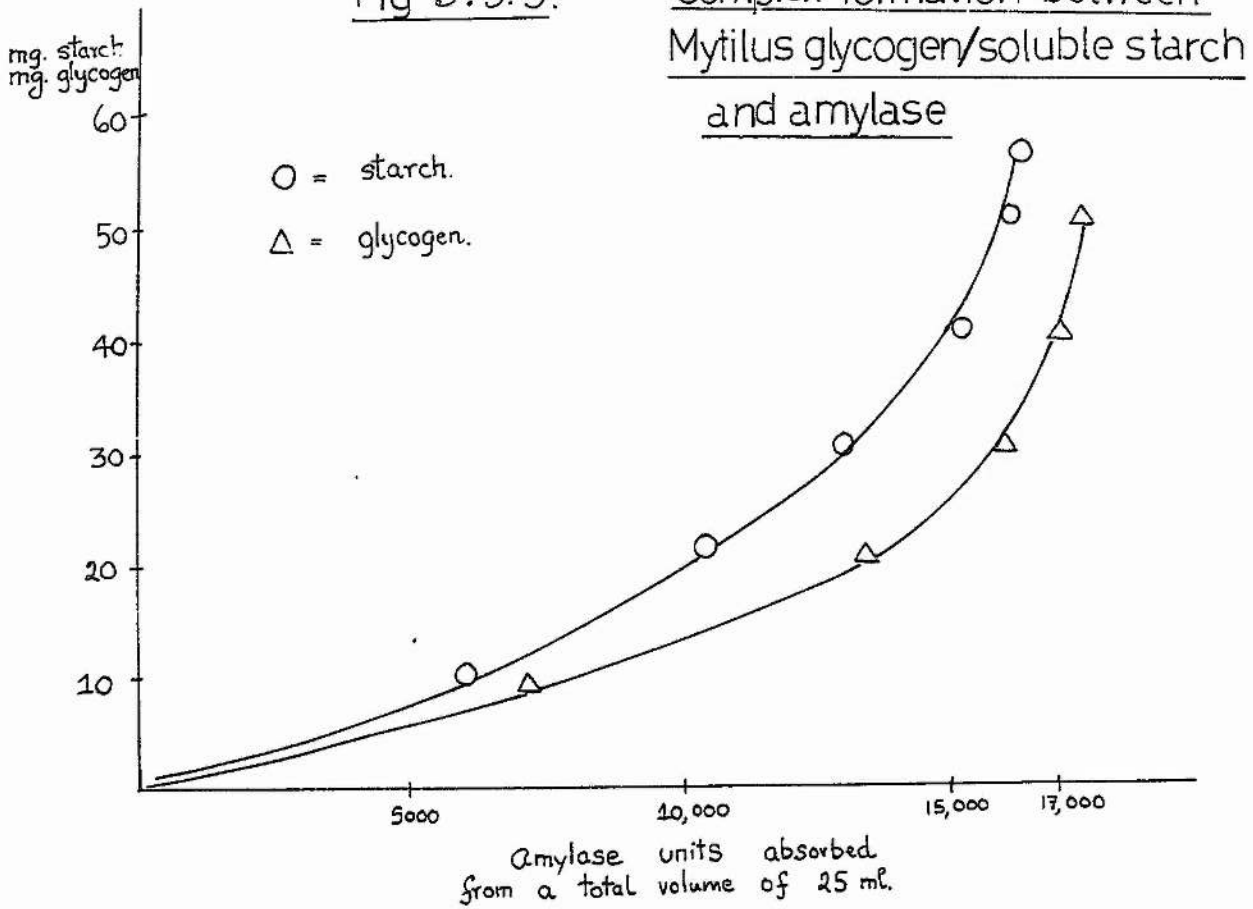
0.50 mg. amylase per 10 mg. glycogen,

0.38 mg. amylase per 10 mg. soluble starch.

3.3.4. Purification factor achieved by glycogen absorption.

The 40-60% ammonium sulphate fraction of B. subtilis amylase (specific activity approximately 1940 units/mg. protein) was subjected to glycogen adsorption purification using an excess of glycogen according to the above procedure described for commercial amylase. After

Fig 3.3.3. Complex formation between Mytilus glycogen/soluble starch and amylase



precipitation of the enzyme-glycogen complex, the supernatant contained less than 5% of the original enzyme activity. The enzyme-glycogen complex was washed with ice-cold 40% ethanol-buffer, resuspended in phosphate buffer at 35°C and incubated for 60 minutes to allow amylase hydrolysis of the glycogen and liberation of free amylase. After glycogen hydrolysis, a small amount of insoluble material remained; this would be the resistant α-macrodextrin core of the glycogen. The specific activity of the amylase was now found to be 13,000 units/mg. glycogen, an increase of some 7-fold over the starting material. The total enzyme loss during this procedure was some 20%, this being the sum of unadsorbed enzyme and enzyme bound to the resistant α-macrodextrin.

3.4. Studies on some characteristics of B. subtilis amylase, its action patterns and comparisons with other amylases

3.4.1. pH-activity profiles

Figure (3.4.1a) shows the pH-activity profile of B. subtilis amylase, purified by glycogen absorption, between pH 3.5 and pH 8.0. The alkaline limb of the curve falls sharply above pH 7.0, while the acid limb falls much more gradually below pH 5.0. The optimum pH for activity is 6.2.

For comparison, figure (3.4.1b) shows the same profile for commercial amylase from B. subtilis (Cambrian Chemical Co. Ltd.), hereafter referred to as 'Cambrian bacterial amylase', between the same pH limits. The pH of optimum activity is 5.7.

3.4.2. Heat stability

Preparations of B. subtilis amylase and Cambrian amylase, both purified by glycogen absorption, were pre-incubated at a series of temperatures between 35°C and 77°C for 30 minutes in 0.1M phosphate buffer pH 5.7.

Fig 3.4.1 (a) pH–activity curve of

B. subtilis amylase

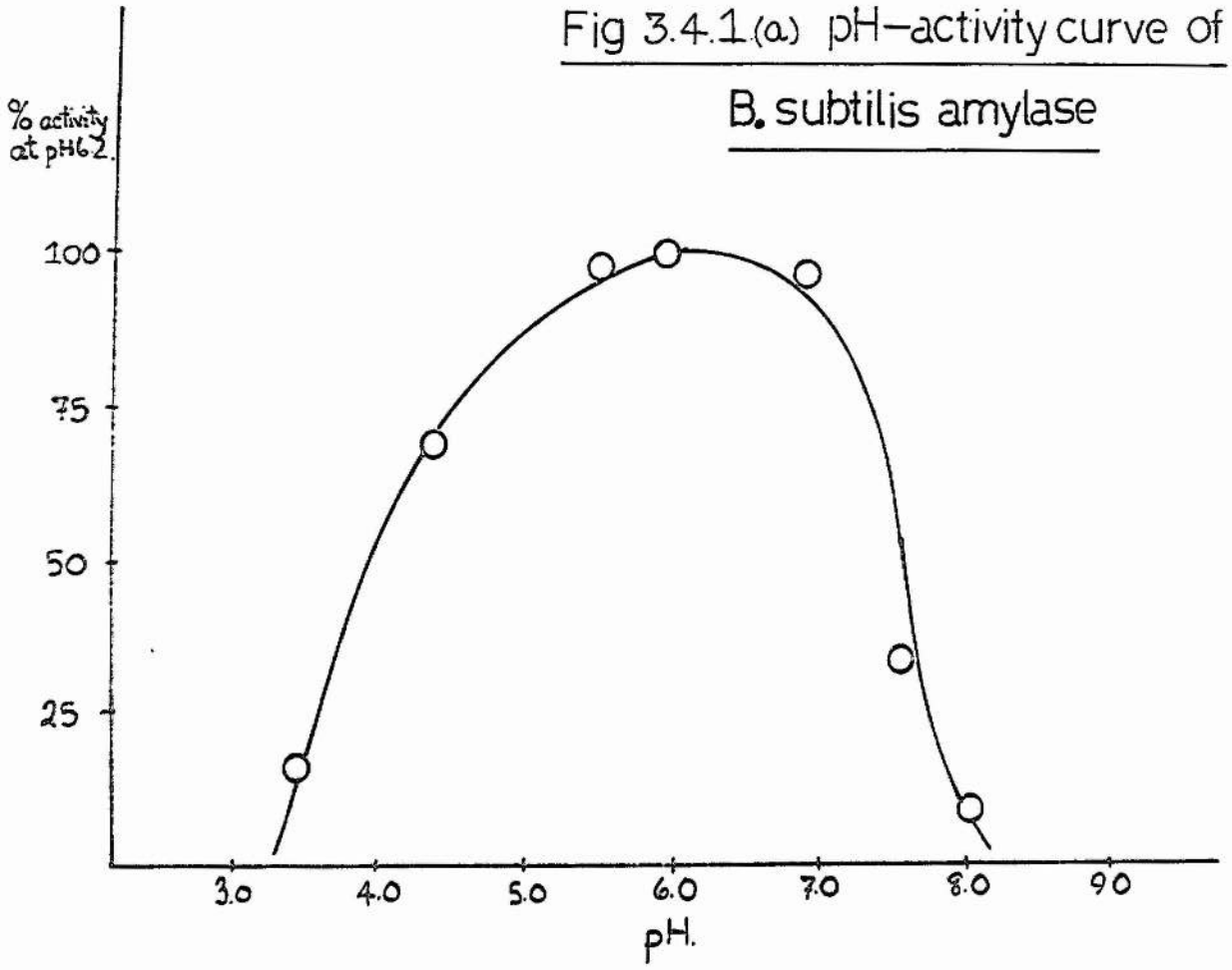


Fig 3.4.1(b) pH-activity curve of Cambrian bacterial amylase

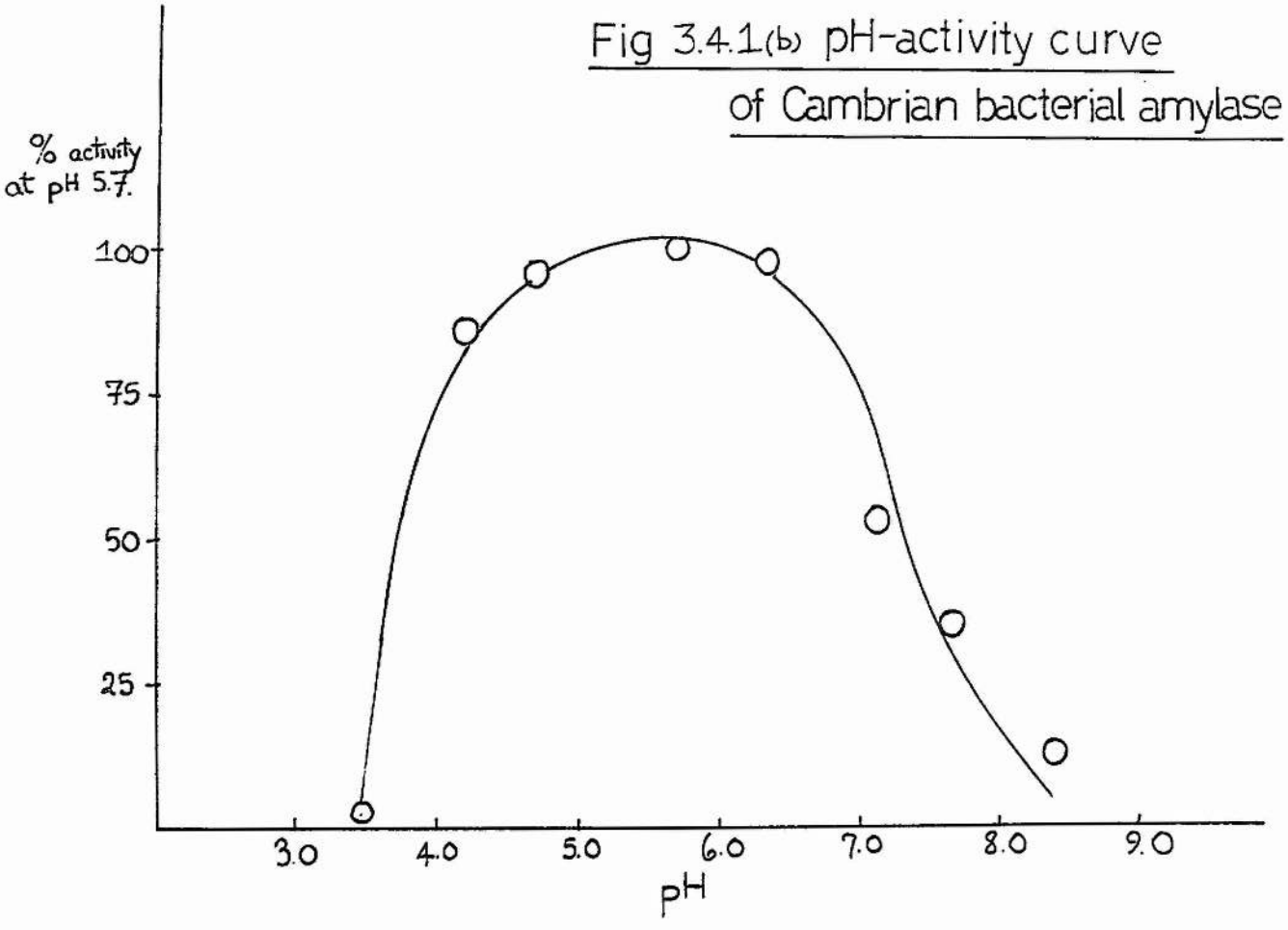
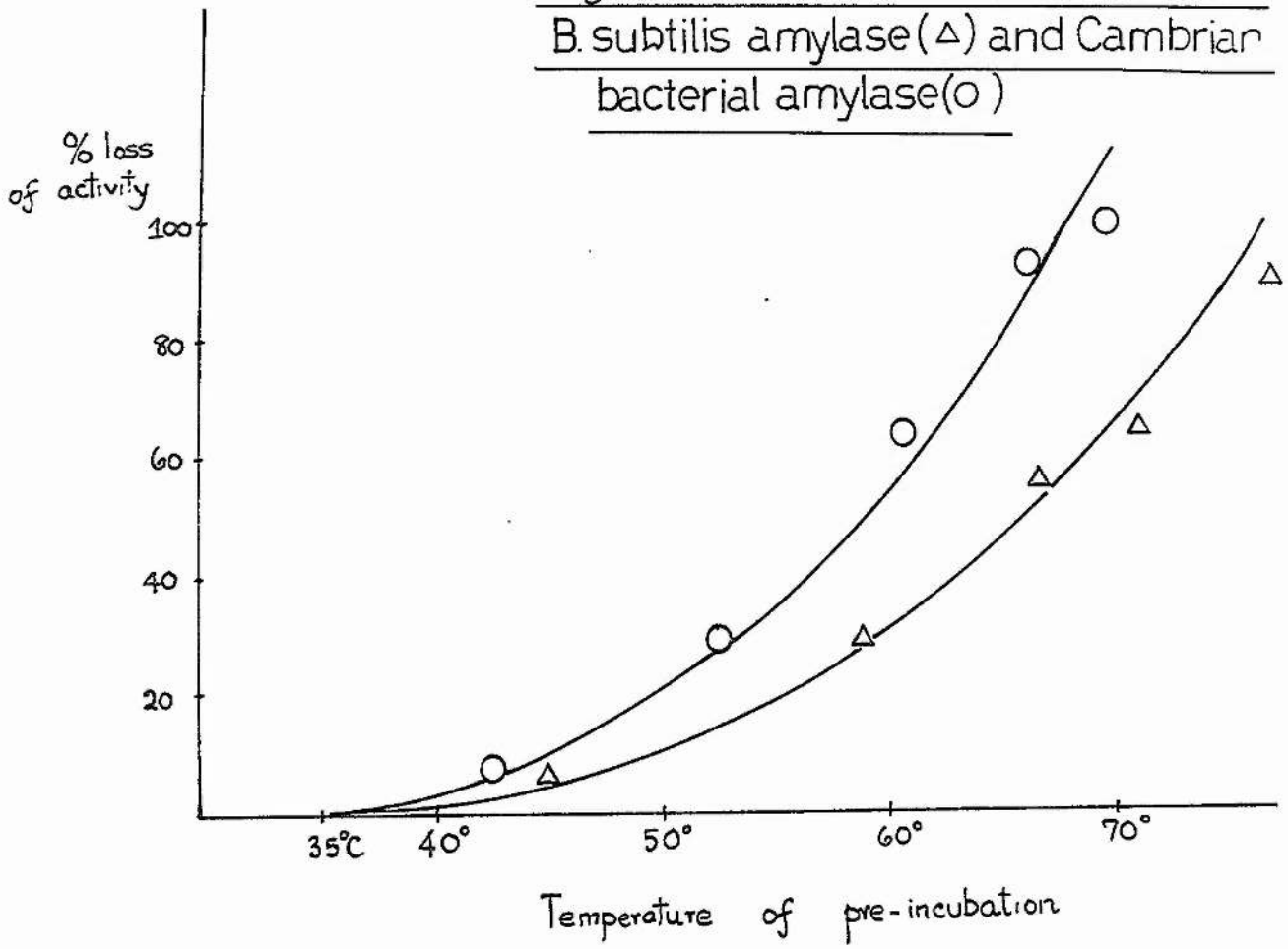


Fig 3.4.2 Thermal inactivation of B. subtilis amylase (△) and Cambrian bacterial amylase (○)



The samples were then rapidly cooled to 35°C and assayed for amylase activity. Figure (3.4.2) shows the loss of activity with increasing temperature of pre-treatment. B. subtilis amylase shows a greater heat stability than Cambrian bacterial amylase. This is likely to be a real difference in that other protein contaminants, which may affect amylase heat stability, should have been removed by glycogen-absorption purification.

3.4.3. Multiplicity of action of B. subtilis amylase and, for comparison, human salivary amylase and Cambrian bacterial amylase

Qualitative differences in the degree of multiple attack by different α-amylases can be easily recognised by recording the fall in blue-value (iodine-staining power) as a function of the increase in reducing power at intervals during the hydrolysis of amylose by the different amylases.

Figure (3.4.3) shows the differences between B. subtilis amylase, human salivary amylase and Cambrian bacterial amylase. It can be seen that B. subtilis amylase displays the highest degree of multiple attack

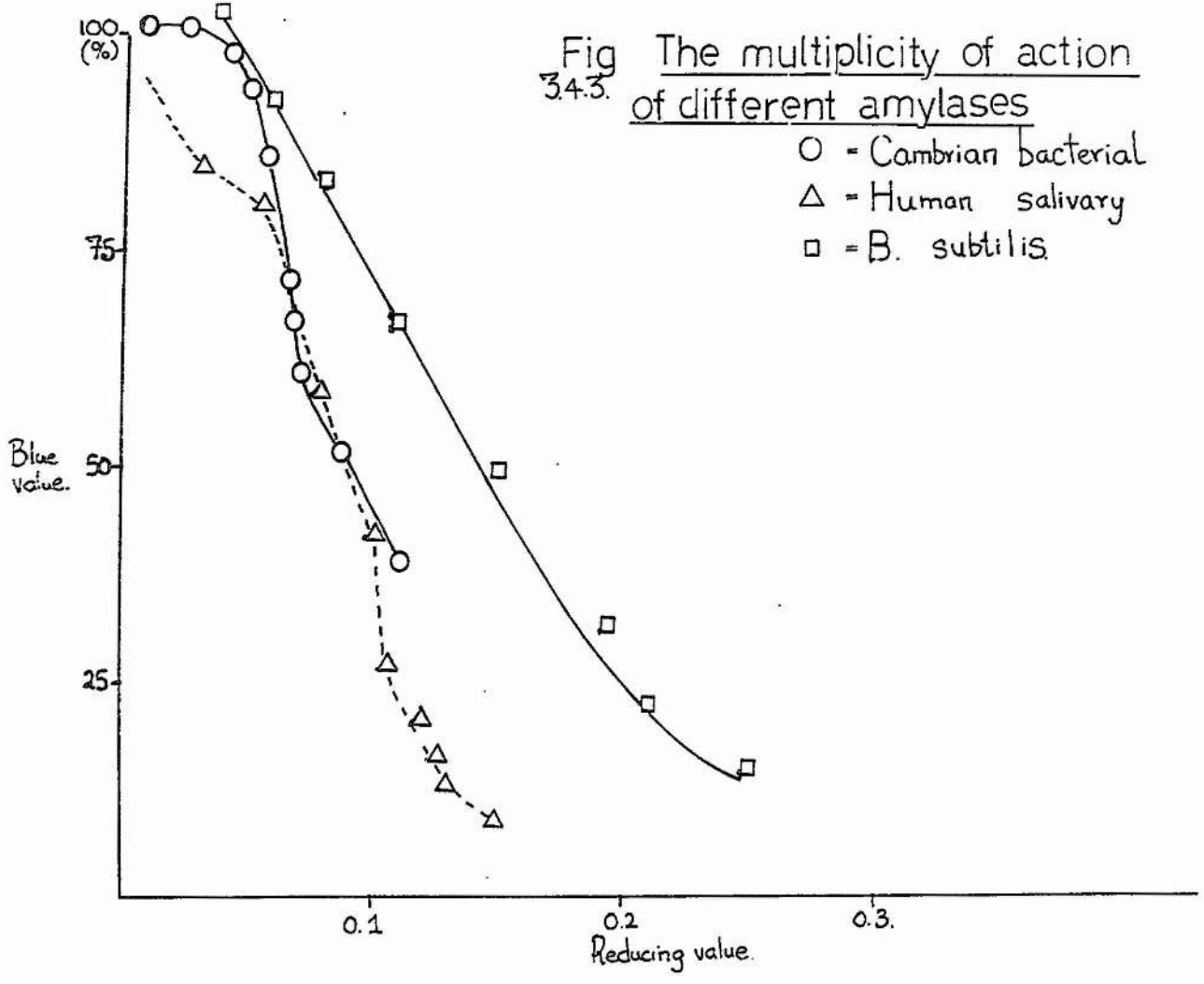


Fig 3.4.3. The multiplicity of action of different amylases

○ = Cambrian bacterial

△ = Human salivary

□ = B. subtilis

of the three enzyme preparations. The other two show essentially similar degrees of multiple attack.

3.4.4. Nishihara (collagen-liberating) activity of various amylase preparations

Samples of freeze-dried deer tendon collagen were treated with samples of

(i) *B. subtilis* amylase

(ii) Commercial crystalline (ex *B. subtilis*) amylase

(iii) Commercial crude (ex *A. oryzae*) amylase

(iv) Cambrian bacterial amylase

for 24 hours at room temperature (23°C) and the amount of collagen which was extractable by subsequent acid extraction was determined by hydroxyproline estimation (collagen contains 14% hydroxyproline)

Table 3.4.4.

Amylase preparation	mg. collagen extractable after enzyme treatment from 50 mg. collagen.
B. subtilis amylase	0.15
Commercial crystalline (ex. *B. subtilis*)	0.15
Commercial crude (ex *A. oryzae*)	0.3
Commercial crude (ex *B. subtilis*)	0.28
Untreated blank	0.15

These results indicate that only crude grades of amylases are effective in increasing the extractability of tendon collagen. The increase in extractability is 100% in tissue treated with crude enzyme compared with untreated tissue or tissue treated with pure amylase preparations which appear totally inactive.

Amylolytic activity of these preparations to which the collagen samples were subjected were adjusted in all cases to approximately 1000 units. Supernatant samples assayed after incubation with collagen showed without exception less than 10% loss of original amylolytic activity.

3.4.5. Hydrolysis of Mytilus glycogen by B. subtilis amylase

10 mg. of Mytilus glycogen were subjected to hydrolysis by 1000 units of *B. subtilis* amylase, purified by glycogen absorption, for 2 hours. The digestion mixture was deionised on Amberlite MB-3 resin and concentrated *in vacuo* prior to chromatography on a 20 x 1.5 cm. column of Sephadex G-25. Figure (3.4.5) shows the elution pattern. The first peak corresponds to a high molecular weight oligosaccharide fraction. On concentration and analysis by ratio of total carbohydrate to reducing carbohydrate it

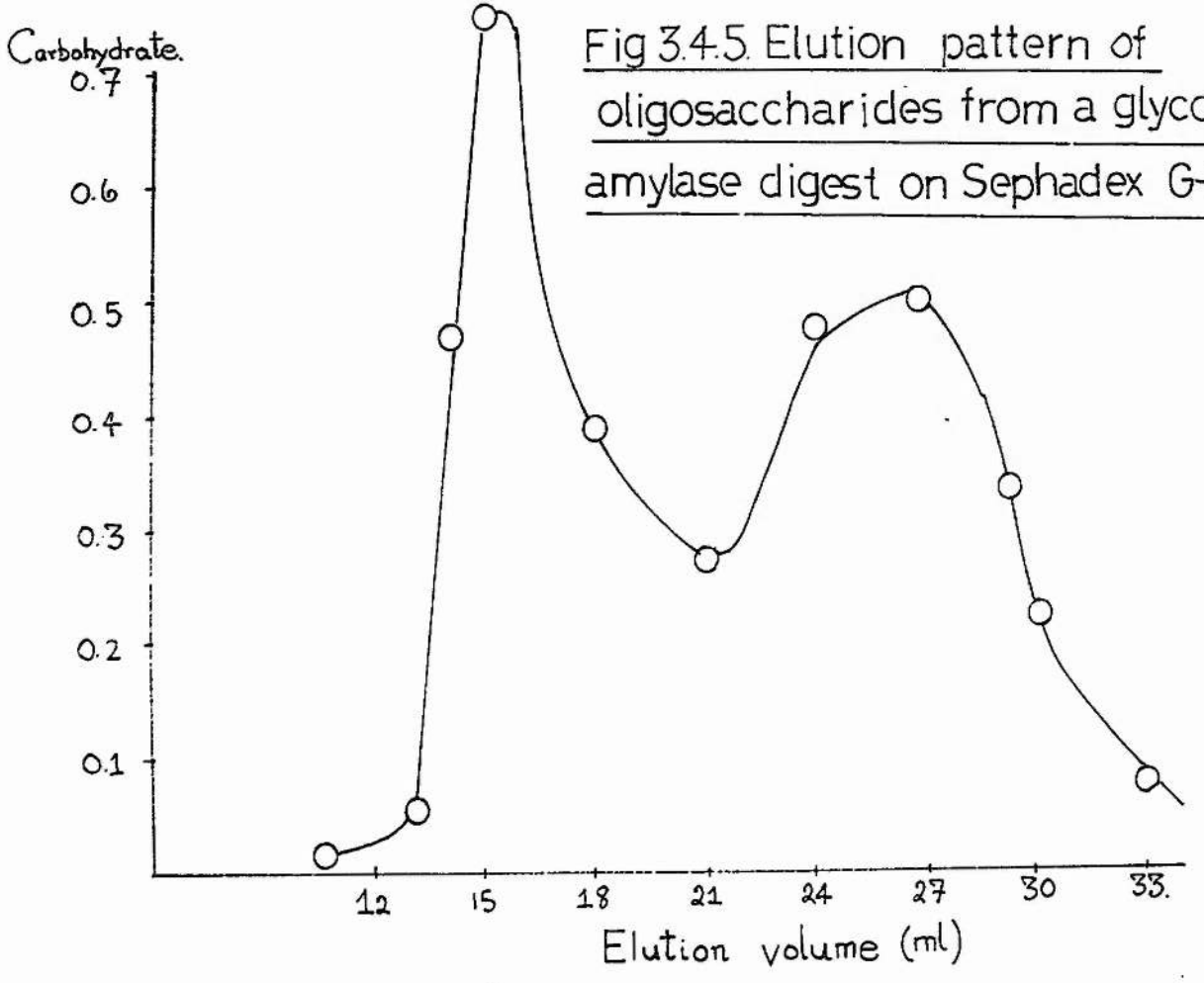


Fig 3.4.5 Elution pattern of oligosaccharides from a glycogen–amylase digest on Sephadex G-25

corresponds to an oligosaccharide of D.P. approximately 20. The smaller peak corresponds in elution volume to maltotriose (the elution volume of an authentic sample of maltotriose had been previously determined experimentally on this column) and also by analysis corresponds to an oligosaccharide of D.P.3.

The oligosaccharide of D.P.20 is probably a soluble α-macrodextrin of glycogen, resistant to further amylase attack.

3.4.6. Oligosaccharide mapping studies of amylases

Oligosaccharide mapping techniques provide a convenient way of studying the hydrolysis of oligosaccharides of the series from maltose (G2) to maltohexaose (G6) by amylases. A partial acid hydrolysate of amylose is developed in one direction on Whatman 3MM paper multisequentially. After drying, the separated oligosaccharides are sprayed *in situ* with a particular amylase and the paper is kept moist and warm for a period of 15 minutes to allow the amylase to act. After a further period of drying, the chromatogram is developed in the second direction. Hence the action

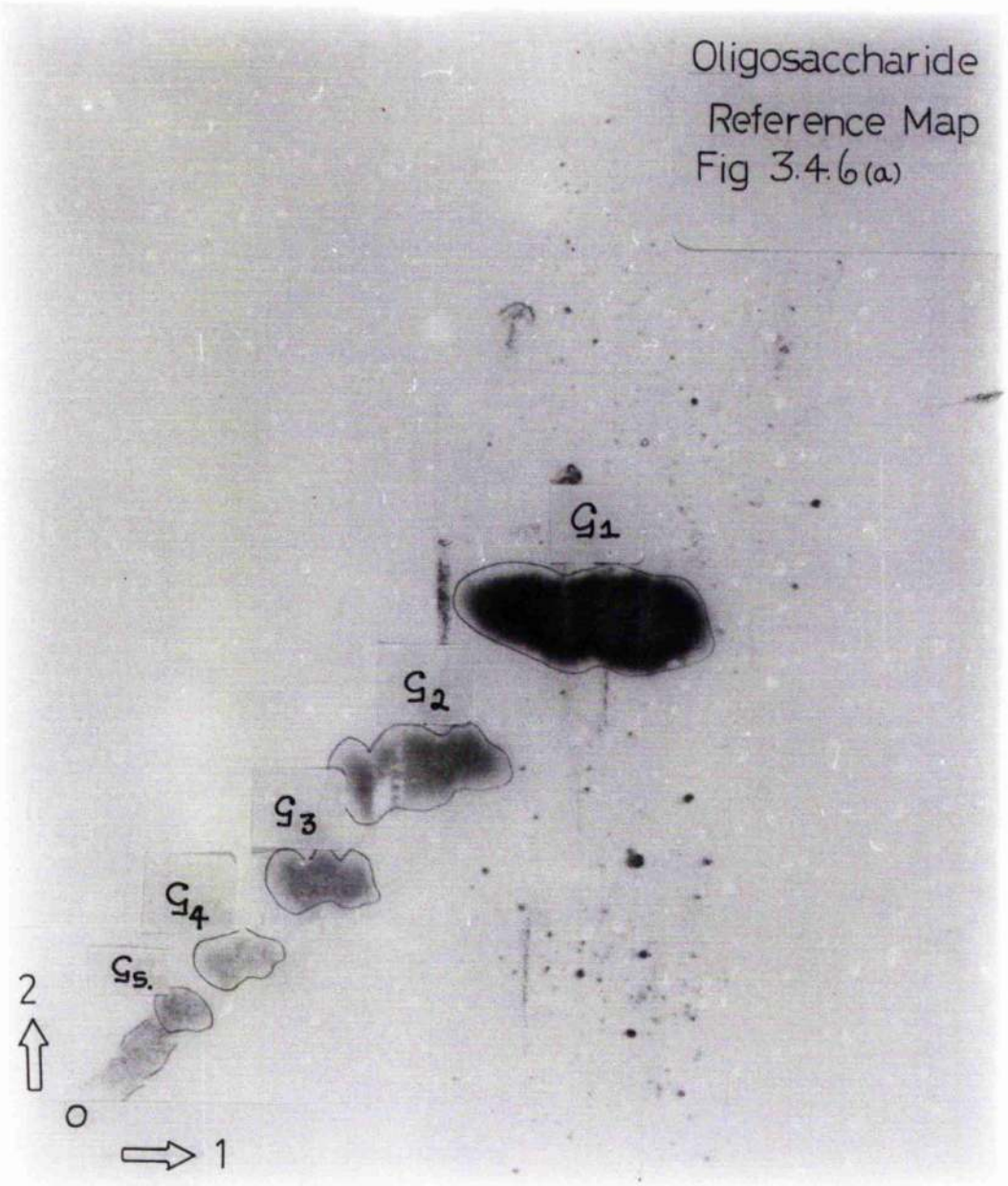
of the amylase on individual oligosaccharides can be studied without the need to isolate the oligosaccharides.

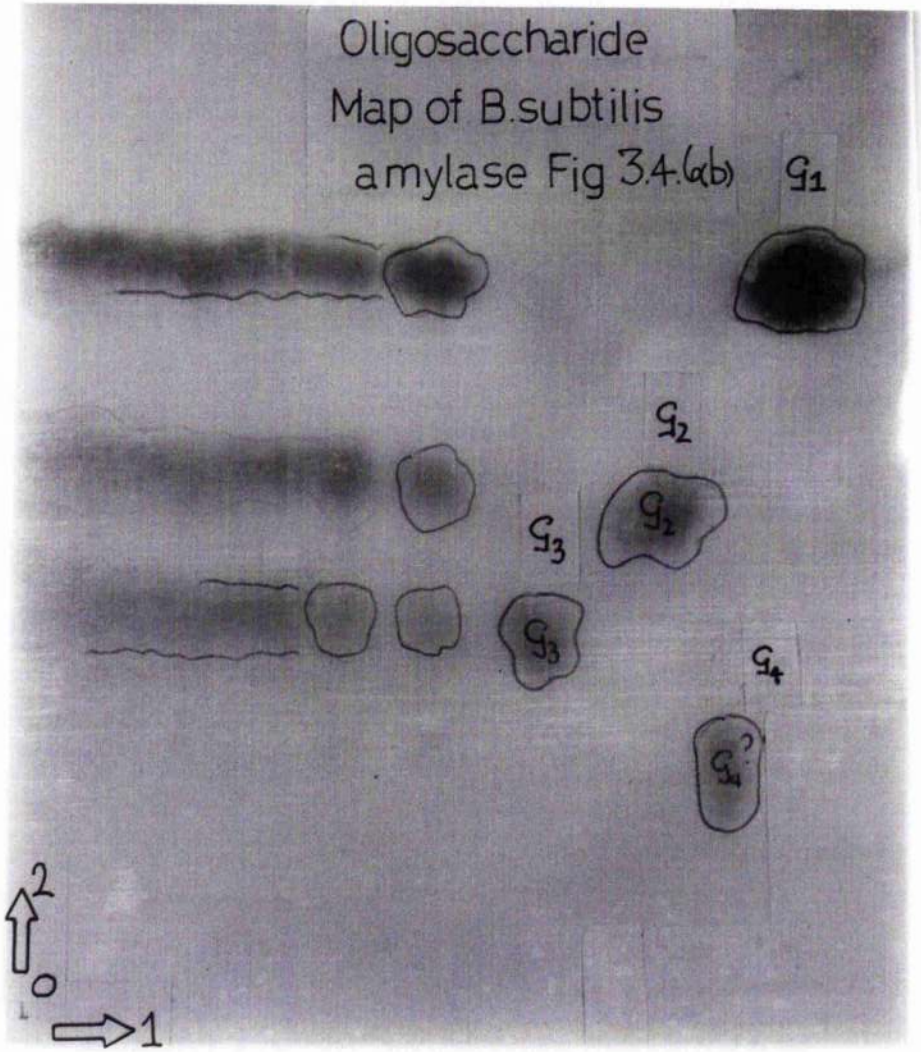
Figure (3.4.6a) is a reference oligosaccharide map in which the spraying stage was omitted.

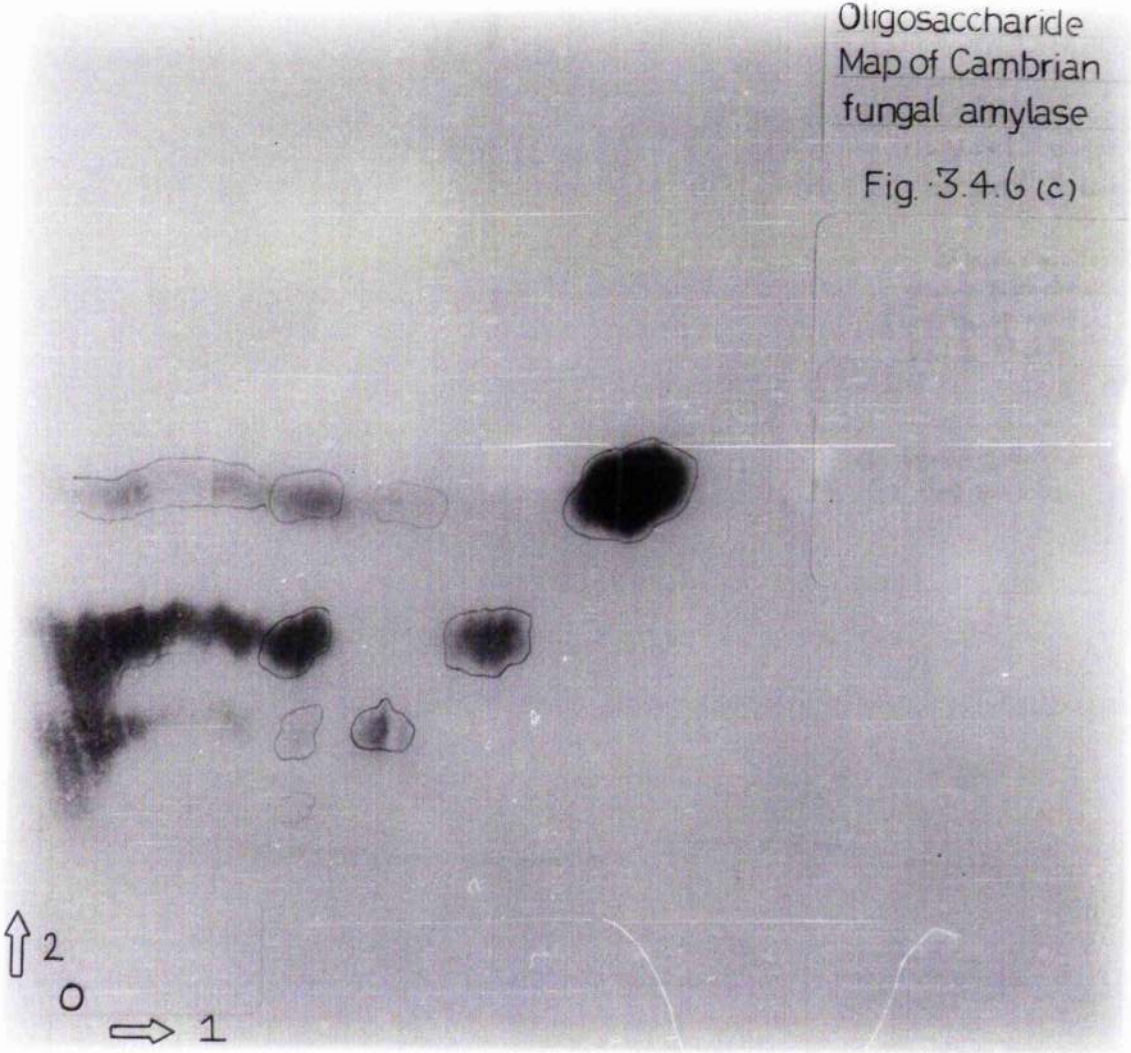
Figure (3.4.6b) shows a map which was sprayed with B. subtilis amylase, 40-60% ammonium sulphate fraction. It is clear that all oligosaccharides greater than G4 have been hydrolysed. It will be noticed that considerable quantities of glucose are produced by this enzyme. This map shows evidence also of G4 synthesis from G2 which would be the result of maltosyl transferase action. Such evidence was not apparent on maps sprayed with glycogen adsorption purified B. subtilis amylase.

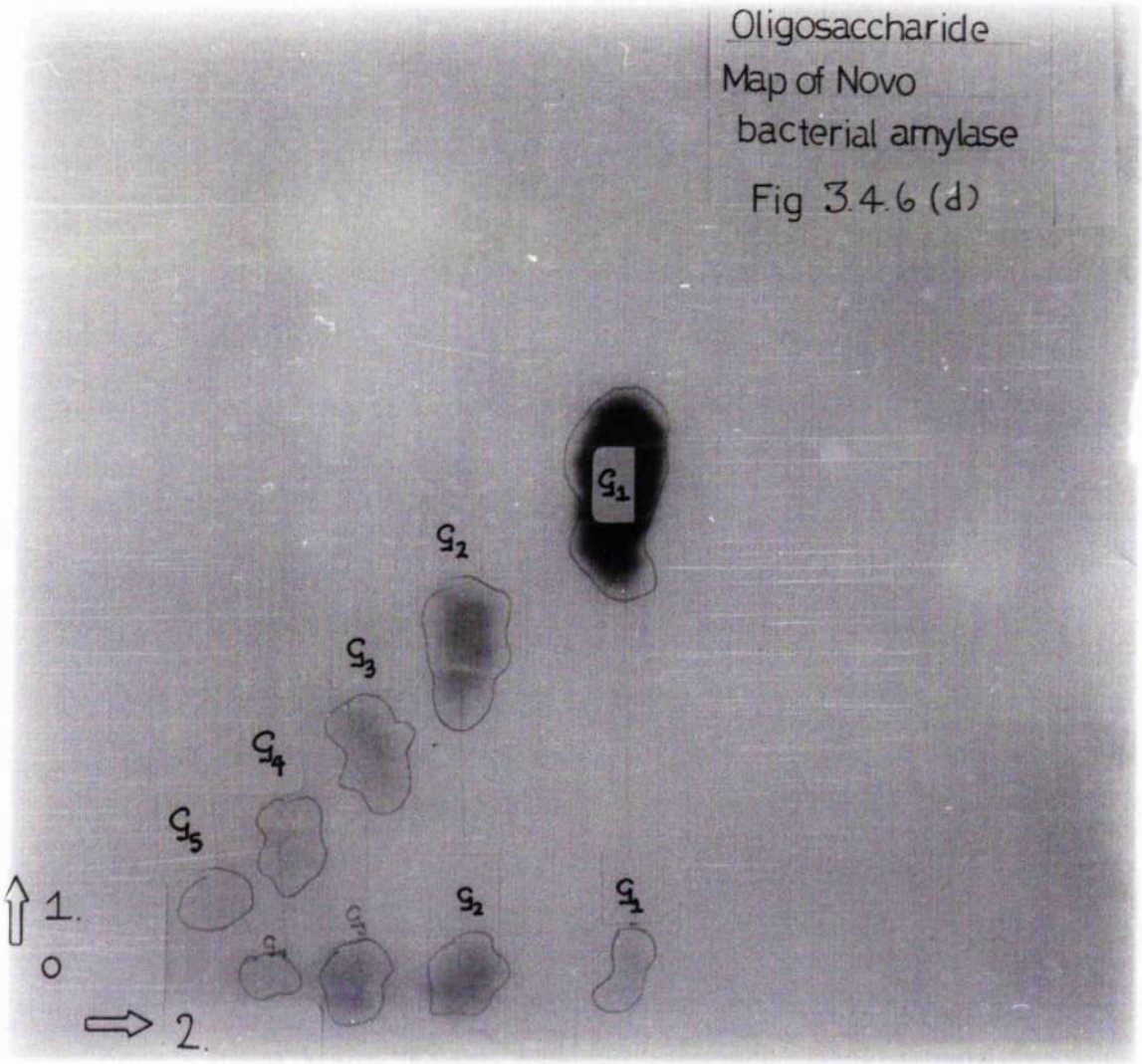
Figure (3.4.6c) shows a map which was sprayed with commercial amylase (ex A.oryzae). The pattern of action is similar to B. subtilis amylase above with the exception that here maltose predominates as the major end product with only small amounts of glucose being produced.

Figure (3.4.6d),(note that the directions of solvent development are reversed in this map), shows a map sprayed with a commercial bacterial amylase (Novo A/S, Denmark). This enzyme appears to have no hydrolytic action on G5 or anything smaller. From higher


Oligosaccharide
Reference Map
Fig 3.4.6 (a)
G1
G2
G3
G4
G5
2
0
1


Oligosaccharide
Map of B.subtilis
amylase Fig 3.4.(a&b)
G1
G2
G2
G3
G3
G4
G4?
2
0
1


Oligosaccharide
Map of Cambrian
fungal amylase
Fig. 3.4.6 (c)
2
O
1


Oligosaccharide
Map of Novo
bacterial amylase
Fig 3.4.6 (d)
G1
G2
G3
G4
G5
1.
0
2.

oligosaccharides, detectably greater amounts of G2 and G3 than glucose are produced.

For the sake of comparison, the activity of all the above enzymes was adjusted to 900-1000 units/ml. before spraying.

3.5. Studies with amylase chemically attached to CM-cellulose and a comparison of some properties of this insoluble amylase and the soluble amylase from which it was derived

Amylase was chemically coupled to CM-cellulose to form a CM-cellulose amylase derivative with amylolytic activity. The amylase used was Cambrian amylase which had been purified by ammonium sulphate fractionation followed by glycogen adsorption. Purification of the crude enzyme was necessary to reduce the attachment of other proteins to the CM-cellulose as the coupling reaction is not in any way specific.

The washing procedure following the chemical coupling ensures the removal of all physically bound protein. The amounts would be small in any case as, at the pH of coupling (8.7), amylase would be negatively charged, and as the CM-cellulose is also negatively charged, there would be little physical attraction between the enzyme and the support.

3.5.1. Degree of protein substitution on CM-cellulose

The amount of protein substituted on CM-cellulose was determined by acid hydrolysis of the product and

estimation of the liberated amino acids by ninhydrin assay using the hydrolysed free enzyme as a standard. A value of 0.58 mg. protein/100 mg. CM-cellulose-amylase was obtained.

3.5.2. Activity of the CM-cellulose-amylase preparation

Amylolytic activity of the insoluble enzyme preparation was determined in the same manner as for the free enzyme except that the assay flasks were shaken at a rapid and constant rate in order that substrate diffusion to the active sites on the insoluble enzyme was not rate limiting (see Hornby, Lilly and Crook, 1966).

The amylolytic activity of the insoluble enzyme was 22 units/100 mg. of CM-cellulose-amylase.

3.5.3. Comparison of the pH-activity profiles of free amylase and CM-cellulose-amylase

Figure (3.5.3) shows the comparative pH-activity profiles of the free enzyme and CM-cellulose-enzyme between pH 3.5 and 8.0. The only difference observed was the displacement of the alkaline limb of the CM-cellulose-enzyme profile by approximately half a pH unit. Activities were measured at 35°C.

Fig 3.5.3. pH–activity curves of CM-cellulose-amylase(Δ) and free amylase(O)

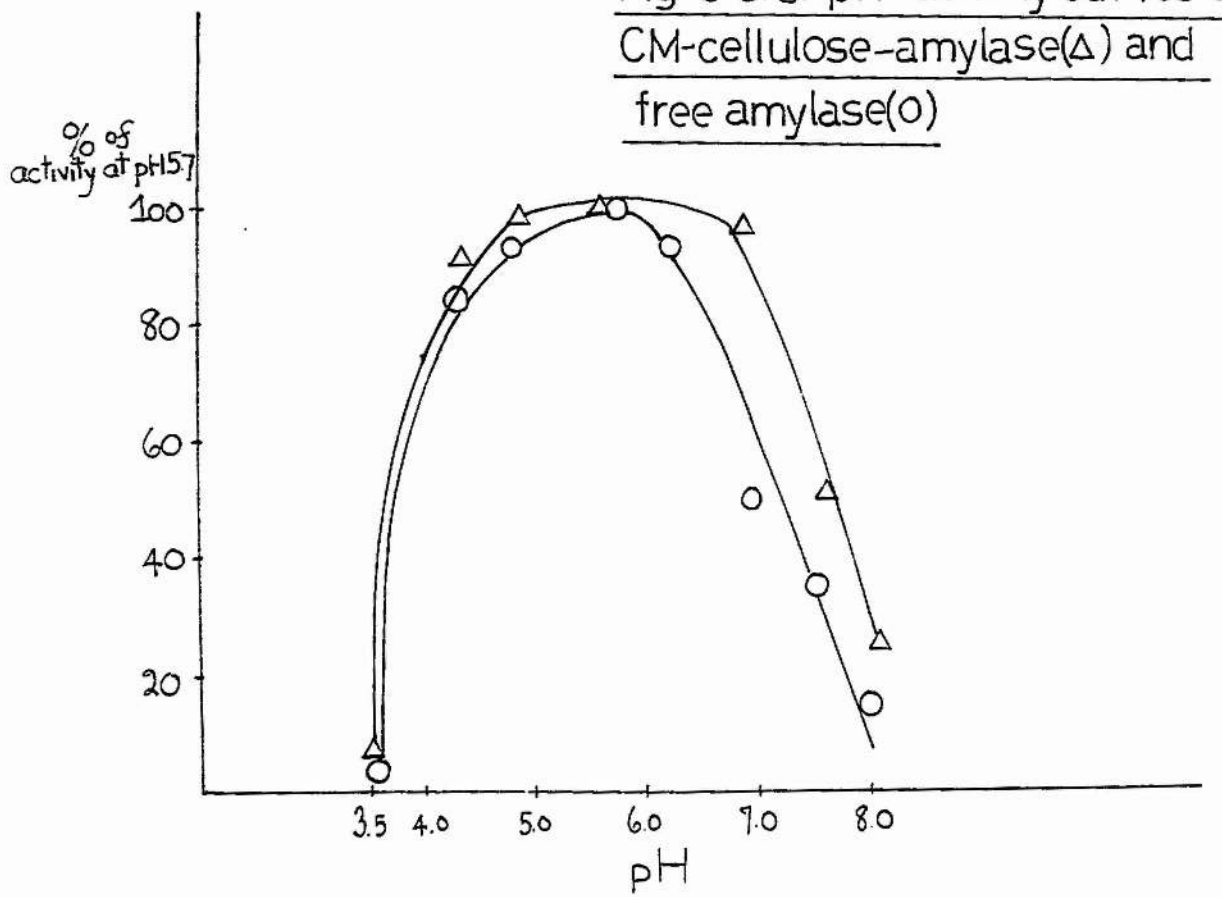
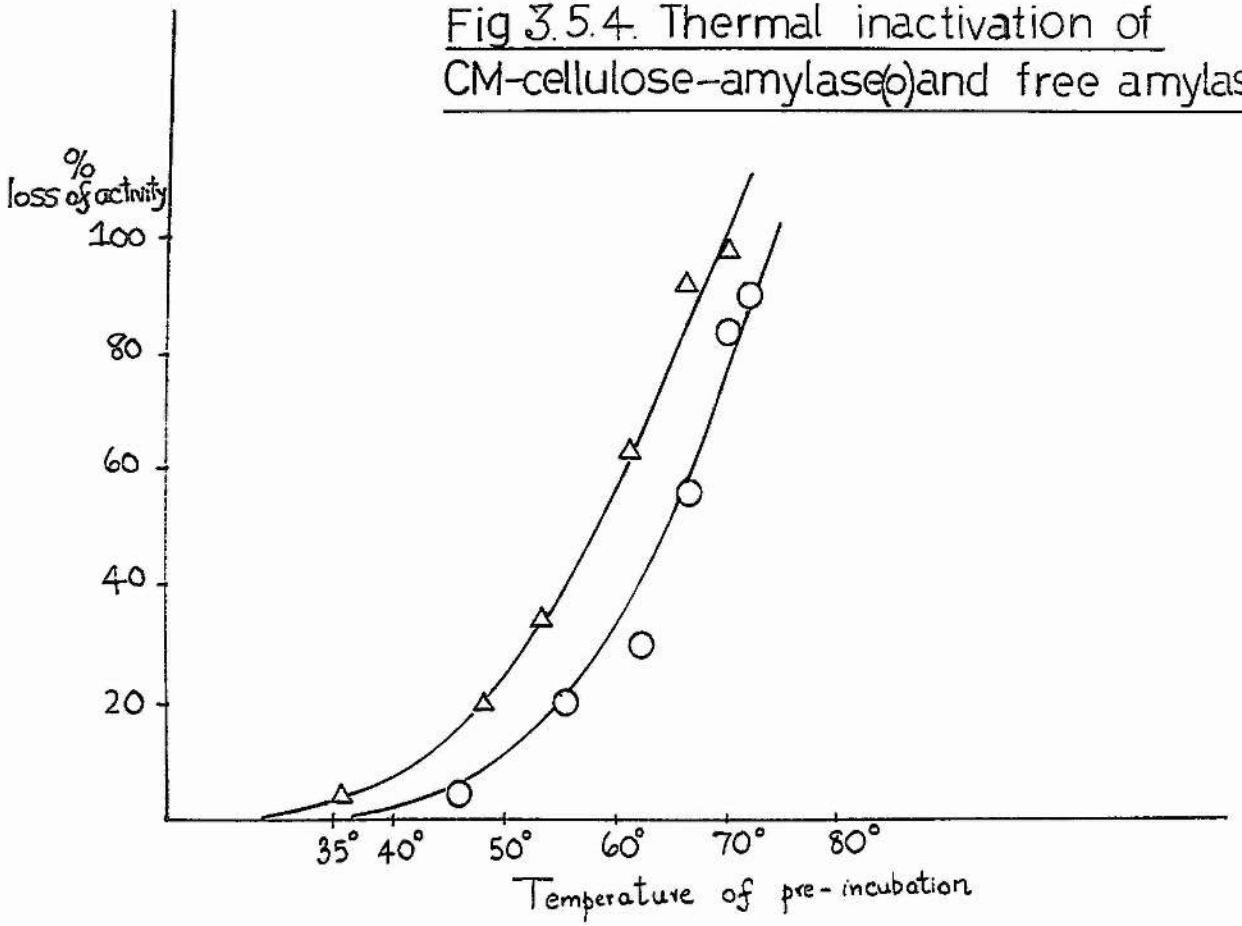


Fig 3.5.4. Thermal inactivation of CM-cellulose-amylase(○) and free amylase(△)



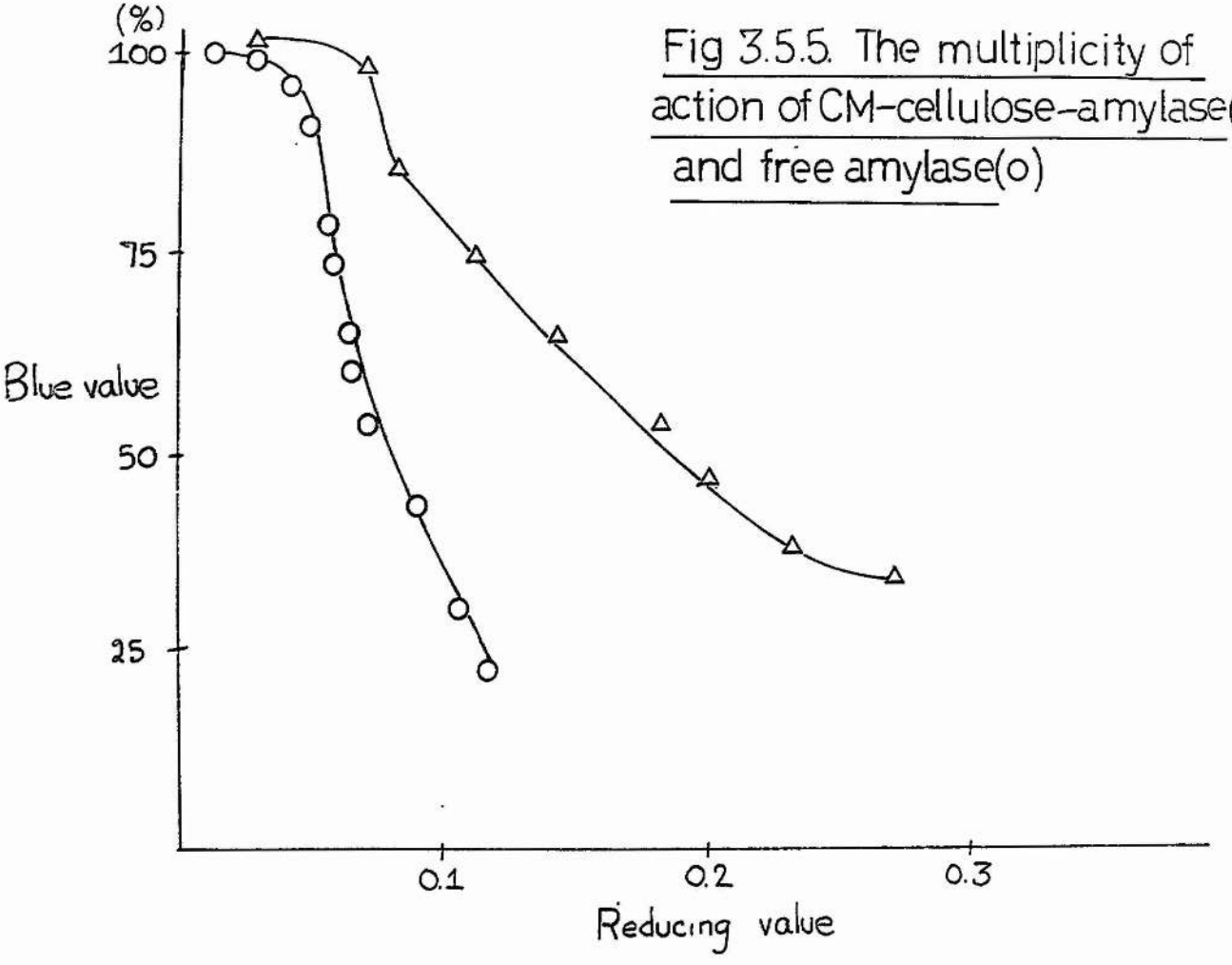


Fig 3.5.5. The multiplicity of action of CM-cellulose-amylase(△) and free amylase(o)

3.5.4. Comparison of heat stability of free amylase and CM-cellulose-amylase

Figure (3.5.4) shows the comparative heat stabilities of the free enzyme and CM-cellulose-amylase. The enzymes were pre-incubated at a series of temperatures for 30 minutes, cooled rapidly and their amylolytic activity assayed at 35°C. It can be seen that attachment of the amylase to the CM-cellulose has increased its heat stability.

CM-cellulose-amylase, stored as a water suspension at 4°C, was found to lose no activity over a period of 1 month. Purified preparations of Cambrian amylase were found to lose as much as 20% activity over a period of a week when stored as a water solution at 4°C.

3.5.5. Comparison of the multiplicity of attack of free amylase and CM-cellulose-amylase

Figure (3.5.5) shows the graphs of loss of blue-value against increase in reducing power for the free enzyme and CM-cellulose-enzyme. Attachment of the amylase to CM-cellulose has markedly increased the degree of multiple attack.

D I S C U S S I O N

4:1. The biosynthesis of bacterial amylase and its release

The results of the present work show that in the case of B. subtilis growing on glucose or sucrose as main carbon source intracellular levels of amylase increase during the initial lag phase, but start decreasing during the logarithmic phase whereas extracellular amylase levels start to increase towards the end of the logarithmic phase. In the case of starch and maltose as carbon supplements, however, a different pattern emerges in that while intracellular amylase levels behave much as above, extracellular enzyme levels parallel growth during the logarithmic phase, indicating a constant rate of release of the enzyme during growth. In the case of starch, where extracellular amylase level was followed for a considerable period after the termination of the logarithmic phase, extracellular levels of enzyme rose linearly for many hours.

These results are in agreement with those of Coleman (1967) who finds that in B. subtilis, low levels of amylase are produced during the growth phase with increased levels

after the termination of the growth phase. Coleman's work gives no data on the intracellular levels.

The early increase in intracellular levels of the enzyme in all cases can be explained on a basis of constitutive enzyme synthesis such as would be expected to take place in bacterial cells during the lag phase before growth. The higher intracellular levels present in the case of cells growing with maltose and starch as carbon supplements can probably be regarded as induced enzyme synthesis when it is remembered that the inocula for these growth experiments were already adapted to the particular carbon source; glucose and sucrose would not be expected to induce amylase synthesis.

The appearance of extracellular amylase early in the logarithmic growth phase, in the case of growth on starch, may well be a cellular mechanism to degrade extracellularly large macromolecules such as starch into low molecular weight products which are more easily assimilated into the cell for catabolism and energy production. Maltose, although not a substrate for amylase, is a major product of amylolytic action and is an inducer of amylase synthesis. The resulting high intracellular amylase levels may trigger off liberation of the enzyme.

Coleman (1967) advances the theory that extracellular enzyme formation is limited during the logarithmic phase of growth because of limitation of m-RNA for extracellular amylase synthesis due to competition at the nucleic acid precursor pool stage. Preferential synthesis of ribosomal protein for rapid growth during logarithmic phase takes place at the expense of extracellular amylase m-RNA synthesis, with a consequent depression of amylase production which, according to Coleman, is not essential for growth. Such a theory would be acceptable for carbon sources the catabolism of which is not dependent on metabolism by amylase, such as glucose or sucrose. However, in the case of starch as the carbon source, amylase synthesis is essential for growth as the starch macromolecule must first be degraded to maltose for further catabolism to take place. Coleman assumes extracellular amylase m-RNA synthesis to be a distinct process from any intracellular amylase m-RNA synthesis. In this case m-RNA synthesis for extracellular amylase might be reduced at the expense of m-RNA synthesis for intracellular amylase. Quite low levels of extracellular amylase would be

sufficient to hydrolyse starch molecules to intermediate-sized oligosaccharides and small oligosaccharides which could then enter the bacterial cell and be further degraded by intracellular amylase. However, it seems obvious that amylase synthesis must take place for either intracellular and/or extracellular use by the bacteria when starch is the sole carbon source as the rate of growth must necessarily depend on the rate of conversion of starch to glucose and maltose which can be degraded in energy-yielding pathways.

A point of interest here stems from the fact that while the initial hydrolysis of starch by amylase is rapid with the formation of a variety of oligosaccharides, the ultimate conversion of these oligosaccharides to glucose and maltose is a relatively slow process requiring, in vitro, high concentrations of amylase. It is possible that, intracellularly, hydrolysis of these oligosaccharides is considerably faster as a result of high local concentrations of intracellular amylase or sterically more favourable conditions for hydrolysis of small oligosaccharides by membrane bound amylase.

Nomura and Hosoda (1958), advancing a different hypothesis, maintain that extracellular liberation of amylase in B. subtilis does not occur during the logarithmic

phase of growth, but only during the stationary phase. They further propose that amylase formation and release occurs only in old cells after the stage of cell multiplication is passed, and this process in old bacterial cells is enhanced by high levels of starch in the medium which protect these unstable cells from osmotic destruction. The present work has shown that with an initial starch level of 1%, the starch concentration falls to 10% or less of its original level before extracellular amylase starts to accumulate in the medium. Extracellular enzyme then continues to rise linearly for many hours after the starch concentration has fallen to zero and can no longer exert a protective effect on the unstable cells.

This raises the question of whether the extracellular amylase is released by cell lysis or regulated secretion of the enzyme. Nomura and Hosoda (1958) isolated a lytic enzyme from cells actively producing and liberating amylase which, they claim, acts on the bacterial cell wall and liberates amylase. In this work it was found that stationary phase cells, which lysed slowly when suspended in phosphate buffer or distilled water, contained cellularly-bound amylase

which was extremely resistant to solubilisation. Ultrasonic disruption of the cells and treatment with synthetic detergent solubilised only very small amounts of amylase, while considerable amylolytic activity still remained in the cell debris after ultrasonic disruption.

It thus seems as if specific secretory mechanisms are involved in the release of amylase from the cell. Two possibilities seem to exist. The first is that amylase is synthesised intracellularly in the protoplast membrane and remains bound in the membrane. When extracellular release is triggered off, the amylase is freed from its membrane-binding site by specific enzymic mechanisms and released as extracellular amylase. Alternatively it seems possible that intracellular amylase designated for eventual release is only weakly bound to membrane and readily liberated, e.g. by lysis of the bacterial cell, while truly intracellular amylase remains tightly membrane-bound. A recent report by Welker and Campbell (1967) clarifies these differing hypotheses of Coleman (1967) and Nomura and Hosoda (1958). Welker and Campbell (1967) have shown that B. subtilis and B. amyloliquefaciens are distinct bacteria which produce immunologically distinct amylases. Nomura and Hosoda's

reported observations on the kinetics of amylase production in B. subtilis were in fact carried out on what they regarded as a strain of B. subtilis, which was termed B. subtilis var amyloliquefaciens. The pattern of amylase production in this latter organism is the same as that reported by Welker and Campbell (1968) for B. amyloliquefaciens. It appears therefore that kinetics of amylase production vary between species and strains of the Bacillaceae which produce amylase.

The experiments performed on the continuous culture of B. subtilis show very low levels of extracellular amylase when cells are growing in the logarithmic phase under steady state conditions. Using the continuous culture technique it is possible to study the liberation of extracellular amylase isolated from any possible effects that there might be from changing levels of metabolites and changes in the physiology of ageing cells as would occur in batch culture.

Under batch culture conditions, starch concentrations fall rapidly as extracellular amylase, even at low levels, hydrolyses it to oligosaccharides. At the same time levels of oligosaccharides may be changing, depending on

the balance between their relatively rapid formation from starch and relatively slower further hydrolysis to smaller sugars. All these factors may have pronounced effects on the synthesis of amylase especially as members of the malto-oligosaccharide series have been shown to be capable of inducing amylase formation. [Welker and Campbell (1963b)]. Under batch culture conditions, a build-up of maltotriose and maltohexaose (B. subtilis amylase is product-specific for these two compounds) may trigger off the linear production of extracellular amylase, which occurs after starch levels have decreased to zero. Under continuous culture conditions, with a starch concentration of 1%, a steady state level of only 0.5 unit/ml. extracellular amylase was observed (See table 3.2.1). Under batch culture with the same medium a final level of 50 units/ml. extracellular amylase was found. The yield of bacteria per gram of starch metabolised in continuous culture, which reflects their ability to convert starch into utilisable metabolites for biosynthesis is of some interest. With starch concentrations of 0.1% and 0.2% the yields are comparable at 0.12 and 0.14 respectively (g. bacteria/g. starch). However, with a 0.3% starch concentration, the yield rises dramatically to 0.6,

indicating a much higher utilisation efficiency. In view of the fact that the extracellular amylase level remains the same when the starch concentration is increased from 0.2% to 0.3%, intracellular levels may be responsible for this seemingly much more efficient conversion of starch into bacterial cells. Intracellular amylase levels have not yet been investigated under continuous growth conditions.

Supernatant amylase formation in washed cell suspensions of B. subtilis was most rapid with maltotriose as the added carbon source when extracellular amylase was formed linearly with time (See fig. 3.1.3a). In the case of amylose and amylopectin there was a lag period before extracellular production increased substantially. A low rate of production was observed with cells incubated in phosphate buffer. After a period, enzyme formation levelled off and this was probably due to a depletion of intracellular amino acid pools. Welker and Campbell (1963c) found that synthesis of amylase in washed cell suspensions of B. stearothermophilus proceeded linearly with respect to time for a period after the addition of an inducer (maltose or methyl α-maltoside) and then levelled off,

at which time the intracellular amino acid pools of the bacteria were found to be depleted in tyrosine, phenylalanine, proline and valine. The present work shows that the addition of a supply of amino acids in the form of a casein hydrolysate gives much elevated levels of extracellular amylase production. In the presence of casein hydrolysate alone, appreciable levels of enzyme were formed, even without any added inducer, but a lag period preceded enzyme formation. Coleman and Elliot (1962) found that while ammonium ions stimulated extracellular amylase formation in washed cells of B. subtilis, the addition of casein hydrolysates tended to inhibit enzyme formation. Fukomoto (1957) however reported stimulation of enzyme formation in B. subtilis by the addition of casein hydrolysates.

Stimulation of enzyme formation by the addition of ammonium ions or casein hydrolysates is evidence for 'de novo' synthesis of amylase in stationary phase cells as against simply release of preformed enzyme which has accumulated intracellularly. In many bacterial cells there is a considerable rate of turnover of cellular protein which is first broken down to amino acids and then resynthesised into other cellular proteins.

In the presence of an inducer, greater amounts of the appropriate enzyme protein are synthesised at the expense of other cellular proteins. Such synthesis will cease when the intracellular amino acid pool is depleted. Eisenstadt and Klein (1961a) studied the synthesis of amylase in washed cells of Pseudomonas saccharophila in the presence of starch, using radioactive tracer techniques. They were able to show increased incorporation of radioactive amino acids into amylase protein in the presence of starch compared with washed cells incubated in the absence of starch.

In the present work, it will be noticed that in all incubations of washed cells of B. subtilis in the presence or absence of inducer a proportion of the cells underwent lysis. Lysis was, however, more rapid in cells suspended in phosphate buffer or distilled water which were producing much lower levels of extracellular amylase. In the case of cells incubated in the presence of inducers lysis was very slow during the phase of amylase liberation and increased only when the rate of amylase liberation decreased towards the end of the experimental period. This, then, is not in accord with Nomura and Hosoda's (1958) hypothesis that

stationary phase cells producing amylase are very unstable and undergo lysis during the actual production of amylase.

4:2 Purification studies on B. subtilis amylase

Purification was attempted from crude culture filtrates prepared from starch — yeast extract — salt media. In purification from large culture volumes, the first object is to reduce the volume of the material being processed rather than achieve a substantial purification factor. Ammonium sulphate precipitation serves this purpose admirably in that 100% saturation of the culture medium with it precipitates all the amylase and the majority of other proteins. Organic solvent precipitation has the disadvantage of requiring large quantities of expensive solvents. Graded fractionation of the ammonium sulphate precipitate can then be more easily carried out and is generally considered to give a better result than direct graded fractionation of the crude culture filtrate.

The initial protein precipitation from the culture filtrate by 100% saturation with ammonium sulphate gave a 1.5-fold purification while the overall purification achieved with initial precipitation, followed by graded fractionation was some 6-fold. The greater part of the amylase protein was recovered in the 40-60% ammonium sulphate fraction with small amounts present in all the

other fractions. This degree of purification seems low when compared with many reports. Robyt and French (1964) reported a 28-fold purification factor with B. polymyxa amylase achieved by a single precipitation from the culture filtrate with 50% ammonium sulphate. Such a high purification factor obtained at this stage of purification probably reflects purification from a starting material containing a high level of non-protein nitrogen, since non-protein specific assays were used viz. optical density determinations at 280 mμ.

Specific adsorption of enzyme to substrate under conditions when the enzyme-substrate complex formed is not reactive and catalysis does not occur is a powerful tool in enzyme purification because of the specificity of the process. This present work shows a further purification of 7-fold when the glycogen adsorption step is applied to the 40-60% ammonium sulphate fraction. This value compares favourably with a 5-fold purification achieved by Loyter and Schramm (1962) in the purification of rat parotid gland amylase.

The characteristics of the adsorption of amylases

to starches and glycogen will depend mainly on two factors:-

1) The specificity of the amylase as regards adsorption on the substrate macromolecule;

2) The source of the starch or glycogen and the treatment it has undergone during extraction and other purification processes.

It has been found that the extent of adsorption of amylase to glycogen is 0.54 mg./10 mg. and to starch 0.31 mg./10 mg. The former value is a factor of 10-fold less than the value reported by Loyter and Schramm (1962) for the adsorption of rat parotid amylase to glycogen. This difference is possibly accounted for by differences in the binding sites for substrate molecules on the two different amylases. It may also be due in part to different molecular fine structures of the glycogens used. The source of the glycogen used by these authors is not mentioned. Shell-fish glycogen was used in the present work and is known to contain regions of dense branching which are highly resistant to penetration and cleavage by amylase [Heller and Schramm (1964)]. Such regions may offer relatively

few points for the adsorption of amylase thus reducing the efficiency of amylase adsorption per molecule of glycogen.

In the absence of phosphorylase and glycogen synthetase, both of which are also adsorbed to glycogen, the protein adsorbed to glycogen by this technique should be pure amylase, provided the amylase itself is not bound to other material.

4:3 Characteristics and mode of action of bacterial amylases

The pH-activity of purified *B. subtilis* amylase and Cambrian bacterial amylase show only slight differences in the optimal pH for activity; that of the former being 6.2 and of the latter 5.7. However, studies of the heat stability of the two amylases show an increased stability of *B. subtilis* amylase over Cambrian bacterial amylase. The former shows a 50% inactivation of enzyme activity after incubation at 66°C. for 30 minutes compared with a similar inactivation after incubation at only 59°C. in the case of Cambrian bacterial amylase.

The substantial qualitative differences in the degree of multiple attack on amylose between *B. subtilis* amylase and Cambrian bacterial amylase indicate that these two enzymes must be distinctly different in their mechanism of attack on amylose and probably therefore of different molecular structure. As Cambrian bacterial amylase is reported to be of Japanese origin it is in all probability a preparation from *B. amyloliquefaciens* rather than *B. subtilis* although labelled as being from

the latter. Welker and Campbell (1967) have shown that a number of commercial amylase preparations claiming to be 'ex B. subtilis' are in fact preparations from B. amyloliquefaciens, a closely related organism which, however, produces an immunologically distinct amylase.

Oligosaccharide maps of the action of the various amylases on maltosaccharides show distinct differences in their ability to hydrolyse small oligosaccharides. Novo bacterial amylase does not hydrolyse oligosaccharides smaller than maltohexaose and the end-products of hydrolysis include greater amounts of maltose than glucose. B. subtilis amylase, on the other hand, produces greater amounts of glucose than maltose and the final end-products include no sugars larger than maltotriose. B. subtilis amylase must attack the terminal glucosidic bond in substrates to liberate substantial amounts of glucose. As the recognised product specificity of B. subtilis amylase is for maltotriose and maltohexaose it is probable that, at the enzyme concentration used in these experiments, maltohexaose was further hydrolysed. In view of the enzymes' attack on terminal glucosidic bonds, one would

have expected maltopentaose to have been a product from this hydrolysis. However, no evidence for a compound corresponding to maltopentaose was apparent on the chromatogram. This may be explained on the basis that the enzyme shows no preferential attack on terminal bonds but as substrate molecules become smaller and smaller the ratio of terminal bonds to total bonds increases and increasing numbers of terminal bonds are hydrolysed, liberating glucose. The oligosaccharide map of Cambrian fungal amylase shows production of more maltose than glucose as among the final end-products and, as with B. subtilis amylase, oligosaccharides larger than maltotriose do not persist as final end-products.

The hydrolysis of shell-fish glycogen by B. subtilis amylase and the attempted separation of the products on Sephadex G-25 showed that maltotriose was a major end-product. The high molecular weight oligosaccharide with a degree of polymerisation of approximately 20 may be an α-macrodextrin of glycogen, which would be expected in view of what is now known of the fine structure of shell-fish glycogen [Heller and Schramm (1964)]. It may, however, be a complex formed from

the α-macrodextrin and amylase molecules as either compound would be eluted with the solvent front from G-25. Such a complex, although of higher molecular weight would still be soluble (See Introduction I:4:5). In view of the possible binding of amylase molecules to dextrins produced during hydrolysis with the consequent increase in molecular weight, gel filtration may not be a totally satisfactory technique for the study of products of amylase hydrolysis.

4:4 The action of bacterial amylases on collagen.

The ability of bacterial amylase preparations to solubilise collagen or to alter the molecule to render it more soluble in dilute acid seems remarkable. The fact that carefully purified preparations of collagen always contain small amounts of carbohydrate material (about 0.5%) suggested that amylase or other carbohydrase might be responsible for this effect. Steer (1966) was able to show that collagen extracted after treatment with bacterial amylase had a lower carbohydrate content than collagen extracted without such pretreatment with the enzyme. Steer also showed that the crude bacterial preparations used in this technique possessed proteolytic activity against collagen, liberating small quantities of peptides, and against haemoglobin. Steer found no evidence of hydroxyproline release, however, in hydrolysates of material solubilised from collagen by the action of bacterial amylase preparations. This suggests that any enzymic activity is not associated with an attack on the triple helix core of the molecule. This view is in keeping with Steven's (1963) finding that collagen solubilised from aged tissues by this

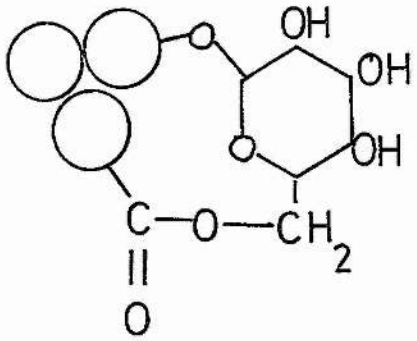
technique is in a highly polymerised state. The present findings on the effect of several bacterial amylase preparations on the solubilisation of deer tendon collagen in dilute acid suggest that, once impurities are removed from the amylase, there is no effect of increased solubilisation of collagen from tissues treated with that preparation. However, Taylor (1966), investigating the effect of bacterial amylase on tendon collagen extractability, made the following surprising discovery. Crude bacterial preparations, which were active in promoting extractability, lost this activity when the amylase was precipitated specifically by rabbit anti-amylase antibody. Taylor tentatively concluded that the concomitant action of amylase, together with another enzyme (unspecified), were responsible for the solubilisation. With regard to the unspecified enzyme there is no basis for further speculation than that it may be either protease or carbohydrase in nature.

With regard to the possible action of amylase alone, it is generally thought that the severing of

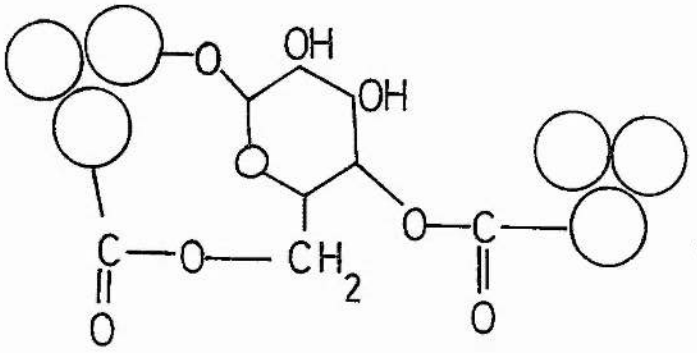
any glucosidic bonds involved in intermolecular or intramolecular cross-linking in the collagen molecule is the most likely site of its action (Steer, 1966). An obvious site for the action of amylase or other carbohydrase would be an oligosaccharide linkage region, such as is present between mucopolysaccharide chains and a protein core in connective tissues, but there is no evidence for a similar covalent bonding of collagen to any other component of connective tissue. The severance of intermolecular links could, however, increase the extractability of collagen. In view of the fact that mammalian collagen usually contains about 0.5% carbohydrate, some of which may be involved in ester cross-linking between triple helices, it may be that amylase is able to hydrolyse certain of these linkages thus rendering the molecule smaller and more soluble.

Hörmann (1960) proposed the illustrated scheme (See Fig. 4:4:1) for intramolecular and intermolecular cross-linking in collagen. The sugar ring is visualised as being associated with hydroxylysine through its glycosidic linkage and with an aspartic acid carboxyl side chain through a chain hydroxyl group.

CROSSLINKING IN COLLAGEN



Procollagen
with
intramolecular bonds



Collagen with
inter— and intramolecular
bonds

[Hörmann, 1960]

Fig 4.4.1

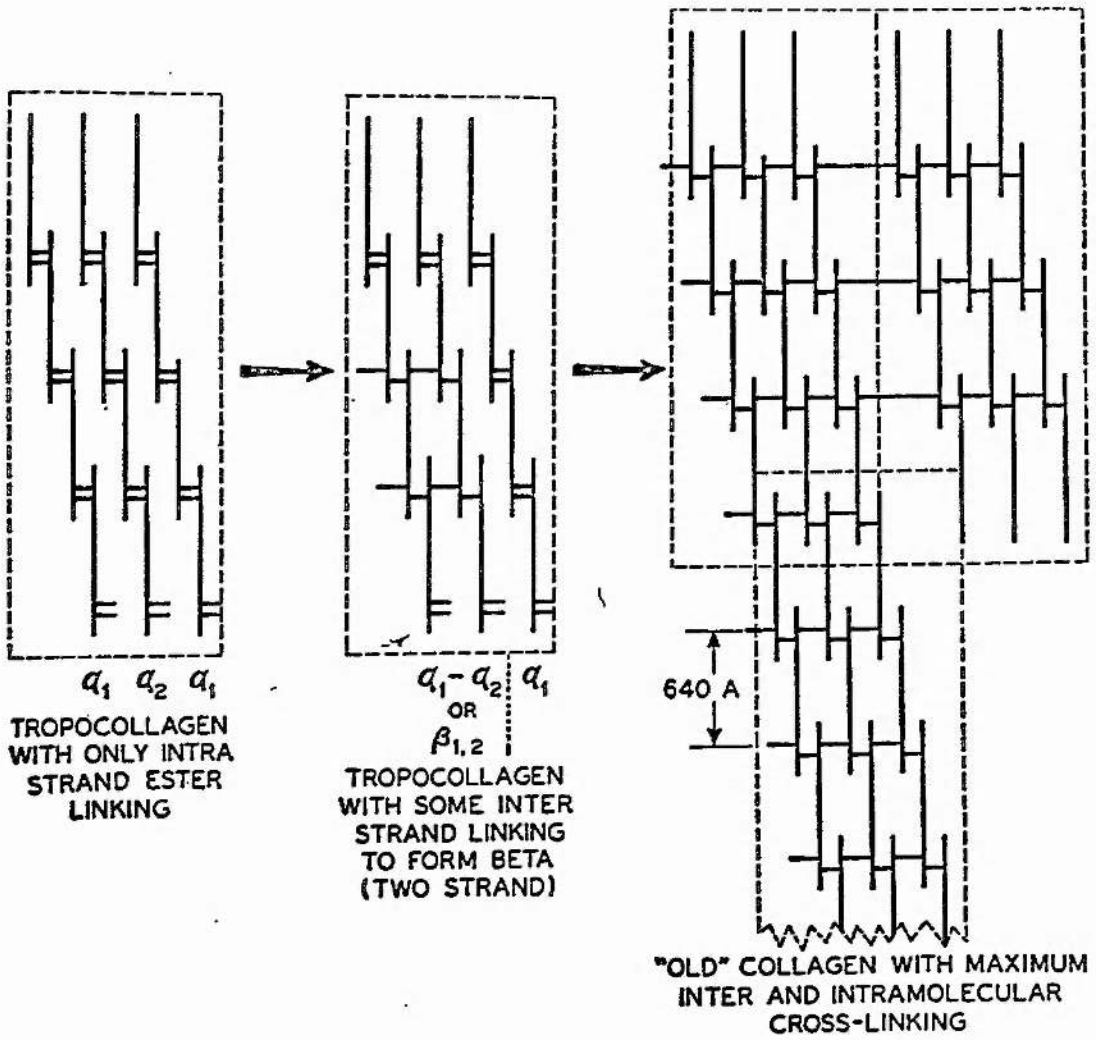

α_1 α_2 α_1
TROPOCOLLAGEN WITH ONLY INTRA STRAND ESTER LINKING
α_1-α_2 α_1
OR
$\beta_{1,2}$
TROPOCOLLAGEN WITH SOME INTER STRAND LINKING TO FORM BETA (TWO STRAND)
640 A
"OLD" COLLAGEN WITH MAXIMUM INTER AND INTRAMOLECULAR CROSS-LINKING

Fig 4.4.2

Figure (4:4:2) shows the proposed arrangement of ester bonding in tropocollagen with the extensive intermolecular cross-linking in more mature tissues. The enzymatic cleavage of these intermolecular bonds would increase the solubility of the tropocollagen in dilute acid. In view of the very slow but finite effect of amylase on small molecules of the trisaccharide type (Walker and Whelan, 1960; Yoshida, 1967) it is possible that hydrolysis could occur on the glycosidic side of the sugar residue, involved in the ester cross-link. Such hydrolysis would, however, be extremely slow and would require the high concentrations of amylase used in this extraction procedure. A further possibility stems from the fact that many strains of B. subtilis produce unspecific β-glucanases (Moscatelli, 1961); however there is no evidence to suggest whether the linkages in collagen are α or β.

As some of the molecular structures proposed for collagen are inconclusive, it is impossible from the present work to draw any firm conclusion regarding the nature and site of action of the enzyme or enzymes responsible for this effect.

4:5 CM-cellulose-α-amylase, an insoluble derivative of amylase

4:5:1. The preparation of CM-cellulose-α-amylase

The chemical attachment of an enzyme to an insoluble support material to produce an insoluble enzyme-support derivative with enzymic activity has been reported for a number of enzymes using a variety of reactive support materials (See Bar-Eli and Katchalski, 1960, 1963; Mitz and Summaria, 1961; Hornby, Lilly and Crook, 1966).

In this present work, α-amylase has been chemically attached to CM-cellulose and the insoluble derivative produced has been found to have amylolytic activity. The coupling reaction of the enzyme to the insoluble cellulose derivative is a type of acyl condensation between the acid azide groups introduced on the cellulose and suitably reactive groups on the protein. According to Frankel-Conrat (1959) the most likely groups to participate in such a reaction are free amino groups of the protein. Junge, Stein, Neuvath and Fisher ~~et al~~. (1959) report the number of available ∈-amino groups of lysine in B. subtilis amylase as 61 residues per 1000 amino acid residues.

These are the groups most probably involved in the attachment of the enzyme to the cellulose support. The number or proportion of such lysine amino groups involved in the attachment is unknown. Free amino groups have been reported as being indispensible for the activity of hog pancreas amylase and Taka-amylase (Radichevitch, 1959), 100% loss of activity occurring when half the free amino groups are substituted. While there is evidence that tyrosyl phenolic groups are required for catalytic activity of B. subtilis amylase (Yamamoto, 1955), there appears to be no information on the requirement for free amino groups for this enzyme.

Consequently, it is not possible to say whether the observed loss of amylolytic activity is due to the unavailability of the amino groups which are involved in the coupling of enzyme to cellulose, or to steric hindrance of substrate molecules by the proximity of the active site of the enzyme to the cellulose macromolecule.

The degree of protein substitution on the cellulose was found to be 0.58 mg. per 100 mg. of product, this value being less than that obtained by Hornby, Lilly and

Crook (1966) for the attachment of ficin to CM-cellulose where values between 1.8 and 4.5 mg. protein per 100 mg. product were obtained.

4:5:2. The properties of CM-cellulose-α-amylase

The amylolytic activity of the preparation was found to be 22 units per 100 mg. CM-cellulose-α-amylase. This value was obtained with assay vessels shaken at 120 oscillations per minute. At lower rates of oscillation the diffusion of amylose to the active sites on the insoluble amylase and the diffusion of products away from them become rate limiting factors in the hydrolysis and lower values are obtained for the activity of the preparation.

The pH-activity profile of the CM-cellulose-α-amylase compared with the free enzyme from which it was prepared shows a displacement of the alkaline limb of the insoluble amylase profile. A similar effect with ficin bound to CM-cellulose, a negatively charged polymer, was reported by Hornby, Lilly and Crook (1966) and ascribed to an effect of the ionised carboxyl groups of the CM-cellulose producing, by proton attraction, a

pH environment around both the support and the active site of the enzyme which is slightly lower than the pH in free solution. The present experiments were carried out in an ionic strength of 0.1M. A higher concentration of salt ions would have reduced this effect as salt cations would have competed with protons for position around the negative carboxyl groups. (See Hornby, 1966).

The increased heat stability of the CM-cellulose amylase preparation when compared with the free amylase, together with its greater stability over long periods at low temperatures can be explained as the result of a restraining influence of the cellulose support on conformational changes in the enzyme protein which might lead to denaturation (See Hornby, 1966). This same restraining influence could also lead to the observed lowering of enzymic activity. The enzyme protein is probably attached to the cellulose support by several bonds which stabilise the secondary and tertiary structure of the protein.

The attachment of amylase to CM-cellulose appears to alter markedly the action pattern of the enzyme. As compared with the free enzyme the cellulose-attached

enzyme has a greatly increased degree of multiple attack. This may readily be explained on the basis of a steric effect due to the close proximity of the active site of the enzyme to the cellulose macromolecule. Diffusion of the large amylose molecule to and from the active site of the enzyme may be slowed by steric hindrance. The main requirement for a high degree of multiple attack is that the average lifetime of an enzyme-substrate complex is long in comparison with the average time taken for each catalytic event. If this is the case, then a number of catalytic events will take place during the lifetime of each enzyme-substrate complex and that number of glucosidic bonds will be severed on the particular chain which is complexed with the enzyme. It is visualised that, between each catalytic event, a rapid sliding re-arrangement occurs exposing a new section of chain to the active sites of the enzyme and in this manner multiple attack on one chain takes place (See Robyt and French, 1963).

Alternatively, in view of the high local concentrations of amylase on the cellulose molecule and the slow rate of diffusion of products away from the macromolecule, the oligosaccharide chains liberated

as in the result of one enzyme-complex formation (during which one or more catalytic events took place) may be further hydrolysed, after formation of new enzyme-substrate complexes by other active sites on the same amylase molecule or a neighbouring amylase molecule in the fixed three-dimensional structure. Such a sequence of events would result in an apparent increase in multiple attack by the experimental procedure employed (plotting blue loss against reducing power increase). The pattern of attack would however be more random as several enzyme-substrate complexes possibly involving more than one enzyme molecule would have been formed despite the involvement of only one amylose chain.

Robyt and French (1967) in their study of the action mechanism of pig pancreatic amylase found that the action of this enzyme changed from multiple attack at pH 8.7 (the optimal pH for this enzyme) to an essentially multichain mechanism of attack at pH 10.6. The possibility exists that since a pH more alkaline than the optimal pH of activity of the enzyme promotes a multichain mechanism of attack, a pH more acid than the optimal may promote the opposite effect, namely a

multiple attack mechanism. The ionisation of the carboxy methyl groups on the cellulose macromolecule can produce a more acid local pH environment (See results section 3:6:3). This electrical charge surrounding the insoluble enzyme may well effect the ionisation of groups on the enzyme protein in the vicinity of the active sites.

An alternative possible explanation of Robyt and French's (1967) observation of multichain attack at alkaline pH may be a change in the molecular configuration of amylose at high pH. Szejtzi, Richter and Augustat et al. (1967) consider that the amylose molecule consists of helical coil regions connected by random chain segments. They have shown that under conditions of high pH, the helical coils undergo a process of coil relaxation with a loss of helical content. Such a molecular transformation could well affect the susceptibility of regions of the amylose molecule to enzymatic attack.

4:5:3. Future developments in this work

The study of amylase attached to CM-cellulose and other insoluble support materials may give valuable

information regarding the activities of intracellularly-bound amylase. The question of the relatively slow rate of hydrolysis of the smaller oligosaccharides by amylase in free solution is of importance when considering that the final conversion of maltotriose and maltohexaose (in the case of B. subtilis) to glucose and maltose, which is very slow, is a necessary step in the overall metabolism of starch for biosynthetic purposes. It may well be the case that intracellular membrane bound amylase is able to hydrolyse such compounds at faster rates than amylase in free solution. A study of how different macromolecular supports attached to amylase molecules can alter the properties of the enzyme as regards hydrolysis of these small oligosaccharides may provide interesting information in this connection.

The nature of the amino acids comprising the active site(s) of B. subtilis amylase are at present unknown. Knowledge of the residues which are involved in the condensation of the enzyme protein with the reactive sites on the cellulose, together with data on the loss of specific activity following attachment of the enzyme to the support material, might throw further light on

the nature of the active sites.

Attachment of the enzyme to different supports with either no ionisable groups or with positively ionisable groups may result in further modification of the action pattern of the enzyme. Studies on the hydrolysis of amylose by insoluble amylases, packed in columns, in which the substrate is perfused through at controlled rates, should be capable of yielding useful information on the various interactions which must take place between the many chain lengths of substrates on which amylase acts.

Hornby (1968) considers that the organisation of water molecules round an enzyme-substrate complex of a hydrolytic enzyme is of considerable importance and could have considerable effects on the rate of hydrolysis. Water is considered, by hydrogen bonding, to have a crystalline type of structure. The greater the stability of such an arrangement, the less reactive the water molecules are to take part in the hydrolysis of the glucosidic bond. The presence of the large cellulose molecule with its hydroxyl groups and consequent vast potential for hydrogen bond formation with water molecules may organise the water molecules in a very

stable arrangement. Such an effect may also be cited as a possible reason for the lowered specific activity of the amylase following attachment to the cellulose.

Biologically active CM-cellulose α-amylase could have considerable industrial potential in the conversion of starches to fermentable sugars. It could be used, because of its enhanced stability, to repeatedly convert large quantities of starches by perfusion through a column of the insoluble enzyme. Fermentable sugars produced in this way would be free of enzyme contamination and by control of the rate of perfusion the extent of conversion of the starch to fermentable sugars could be regulated.

5:0:0 SUMMARY

1. The biosynthesis and extracellular liberation of α-amylase by cultures of B. subtilis in the presence of a number of carbon sources has been investigated.

2. Small amounts of extracellular amylase were released before the end of the logarithmic phase of growth.

3. Under continuous culture growth conditions, only very small amounts of extracellular amylase were produced during growth on starch-containing media.

4. B. subtilis amylase can be effectively purified by specific complex formation with glycogen.

5. Some of the properties of B. subtilis amylase and commercial amylases claimed to be preparations from B. subtilis have been investigated and the two shown to differ appreciably with respect to heat stability and pattern of attack on amylose.

6. An insoluble derivative of amylase, CM-cellulose-α-amylase, has been prepared and the combination of the enzyme with the cellulose macromolecule has been shown to alter the characteristics and mode of action of the enzyme. An attempt has been made to interpret these effects.

REFERENCES

Powar and Jagannathan
Babbar, ~~et al.~~ (1962) Biochem. Biophys. Acta. 65, 347

Bar-Eli and Katchalski (1960) Nature, London, 188, 856.

Bar-Eli and Katchalski (1963) J. Biol. Chem. 238, 1690.

Bucher (1947) Biochem. Biophys. Acta. 10, 121

Campbell (1955) Arch. Biochem. 54, 154
Caldwell (1952) J. Am. Chem. Soc. 74, 4033.
Cocking and Yemm (1954) Biochem. J. 58, xii

Coleman and Elliot (1962) Biochem. J. 83, 256

Coleman (1967) J. Gen. Microbiol. 49, 421

De Pinto and Campbell (1968b) Biochemistry 7, 121

De Pinto and Campbell (1968a) Biochemistry 7, 114

Eisenstadt and Klein (1961) J. Bacteriol. 82, 798

Eisenstadt and Klein (1961a) Biochem. Biophys. Acta. 44, 206

Erlanger (1958) Biochem. Biophys. Acta. 27, 646

Frankel-Conrat (1959) In the Enzymes, vol.1, p589, ed. Boyer et al.

French (1957) Advances in Carbohydrate Chem. 12

Fukomoto (1957) Proc. Int. Symp. Enz. Chem. 359, Tokyo.

Glaeser and Mel (1966) Arch. Biochem. 113, 77

Hagihara (1951) Proc. Imp. Acad. Jap. 27, 346

Heller and Schramm (1964) Biochem. Biophys. Acta. 81, 96

Hodge and Hofreiter (1962) In 'Methods in Carbohydrate Chemistry' 1, 380. Ed. Whistler, Acad. Pres

Holmberg (1933) Biochem. Z. 258, 134

Hormann (1960) Beitr. Silikose-Forsch. Sonderband 4, 305

Hornby (1966) Ph.D. thesis, University of London.

Hornby, Lilly and Crook (1966) Biochem. J. 98, 420.

Hornby (1968) Personal communication

Jungo, Stein, Neuvalth and Fisher et al. (1959) J. Biol. Chem. 234, 556

Keen and Bechard (1946) Arch. Biochem. 10, 41.

Kornberg (1950) J. Biol. Chem. 182, 779

Kung (1953) J. Amer. Chem. Soc. 75, 5548

Leloir and Goldenberg (1962) J. Biol. Chem. 235, 919

Levitski, Heller and Schramm (1964) Biochem. Biophys. Acta. 81, 101

Loyter and Schramm (1962) Biochem. Biophys. Acta. 65, 200

Maley, McGavrachan and Delljiacco et al. (1966) Biochem. Biophys. Res. Comm. 25, 85

Manning and Campbell (1961) J. Biol. Chem. 236, 2925

Meyer (1947) Experentia 3, 411

Mitz and Summaria (1961) Nature, London, 189, 576

Moscatelli (1961) J. Biol. Chem. 236, 2858

Nelson (1944) J. Biol. Chem. 153, 375

Nishihara (1963) Japanese patent No. 9295/63

Nomura and Hosoda (1958) Biochem. Biophys. Acta. 29, 161

Nomura (1958)	J. Biochem. (Japan) 45, 737
Nomura, Maruo and Akabori (1956)	J. Biochem. (Japan) 43, 143
Pazur (1962)	In 'Methods in Carbohydrate Chemistry 1, 537, Ed. Whistler, Acad. Pres
Peltier and Bechard (1945)	J. Bacteriol. 50, 711
Radichevitch (1969)	J. Amer. Chem. Soc. 81, 2845
Rickenberg and Lester (1955)	J. Gen. Microbiol. 13, 279
Robyt and French (1963)	Arch. Biochem. 100, 451
Robyt and French (1964)	Arch. Biochem. 104, 338
Robyt and French (1967)	Arch. Biochem. 122, 8
Schwimmer and Balls (1949a)	J. Biol. Chem. 179, 1063
Schwimmer and Balls (1949b)	J. Biol. Chem. 180, 183
Schoch (1961)	Proc. Ann. Meet. Amer. Soc. Brew. Chem. p.83
Seaman, Jackson and Uhlenbruck (1967)	Arch. Biochem. 122 605.
Somogyi (1952)	J. Biol. Chem. 195, 19
Starkenstein (1910)	Biochem. Z. 24, 210
Steer (1966)	Ph.D. thesis, St. Andrews University
Stegemann (1958)	Z. Physiol. Chem. 311, 41
Stein and Fisher (1961)	in Biochemical Preps. 8, 34
Steven (1964)	Ann. Rheum. Diseases 23, 600
Street (1963)	in 'Methods in Enzymatic Analysis p.858, Ed. Bergmeyer, Acad. Press.

Street and Close (1958) Clin. Chim. Acta 3, 476

Sutherland and Wacilait (1960) J. Biol. Chem. 218, 45

Szejtzi Richter and Augustat (1967) Biopolymers 5, 5

Taylor (1965) Honours B.Sc. Thesis, St. Andrews University

Tilden and Hudson (1942) J. Bacteriol. 43, 527

Trevelyan Proctor and Harrison. (1950) Nature, London, 166, 444

Walker and Whelan (1960) Biochem. J. 76, 257

Welker and Campbell (1963a) J. Bacteriol. 86, 681

Welker and Campbell (1963b) J. Bacteriol. 86, 687

Welker and Campbell (1963c) J. Bacteriol. 86, 1196

Welker and Campbell (1963d) J. Bacteriol. 86, 1202

Welker and Campbell (1967) J. Bacteriol. 94, 1124

Wolfrom and Thompson (1962) In 'Methods of Carbohydra Chemistry' 1, 334, Ed. Whistler, Acad. Pres

Yamamoto (1955) Bull. Agri. Chem. Soc. Jap. 19, 131.

Yoshida (1967) J. Biochem. (Japan) 62, 43